Factors Affecting Hydrogen Production and Consumption by Human Fecal Flora

The Critical Roles of Hydrogen Tension and Methanogenesis

Alessandra Strocchi and Michael D. Levitt

Research Service, Veterans Administration Medical Center, and Department of Medicine,
University of Minnesota, Minneapolis, Minnesota 55417

Abstract

We studied the influence of hydrogen tension (PH_2) and methanogenesis on H_2 production and consumption by human fecal bacteria. Hydrogen consumption varied directly with PH_2 , and methanogenic feces consumed H_2 far more rapidly than did nonmethanogenic feces. At low PH_2 , H_2 production greatly exceeded consumption and there was negligible accumulation of the products of H_2 catabolism, methane and sulfide. Thus, incubation at low PH_2 allowed the first reported measurements of absolute as opposed to net H_2 production. Feces incubated at high and intermediate PH_2 had a net H_2 production of only 1/900 and 1/64 of absolute production. Glucose fermentation by fecal bacteria yielded an absolute H_2 production of 80 ml/g, a value far in excess of that excreted by volunteers ingesting lactulose. We conclude that most H_2 produced by colonic bacteria is consumed and methanogenesis and fecal stirring (via its influence on fecal PH_2) are critical determinants of H_2 consumption and, hence, net H_2 production. Study of fecal samples from four subjects with low breath H_2 excretion after lactulose showed that absolute H_2 production was normal, and the low H_2 excretion apparently reflected increased consumption due to rapid methanogenesis (two subjects) and decreased luminal stirring (two subjects). (*J. Clin. Invest.* 1992; 89:1304–1311.)

Key words: colonic flora • intestinal gas • methane

Introduction

Understanding the factors that influence hydrogen (H_2) production and excretion could have important clinical implications and provide basic information on the regulation of the colonic ecosystem. Flatus may contain very high concentrations of H_2 (1), and therapeutic maneuvers that reduce H_2 production should benefit patients with flatulence. In addition, a better understanding of H_2 physiology should allow for more accurate interpretation of the H_2 breath tests that are being widely used for the study of carbohydrate malabsorption, small-bowel transit time, and bacterial overgrowth. At a more basic level, H_2 has been shown to be an important substrate for several species of colonic bacteria, and knowledge of the metabolism of this gas could yield new insights into the complex interactions of fecal bacteria.

This work was presented in part to the American Gastroenterological Association in 1990.

Address reprint requests to Dr. Levitt, ACOS for Research, VAMC, 1 Veterans Drive, Minneapolis, MN 55417.

Received for publication 24 August 1990 and in revised form 12 November 1991.

The Journal of Clinical Investigation, Inc.
Volume 89, April 1992, 1304–1311

Bacterial fermentation reactions are the sole source of H_2 production in the intestine, and carbohydrates, both of exogenous and endogenous origin, are the preferred substrate for these reactions (2, 3). A proposed stoichiometry for carbohydrate fermentation by colonic bacteria suggests that an enormous volume of H_2 should be produced from a relatively small amount of substrate (4, 5). However, H_2 excretion via the lungs and the anus (the only excretory routes of H_2) after ingestion of a nonabsorbed carbohydrate is far less than predicted from the theoretical calculation (6). Because H_2 cannot be metabolized by mammalian cells, the relatively low H_2 excretion suggests that the proposed stoichiometry is incorrect or that large amounts of H_2 are consumed in the colon. Bacterial H_2 consumption has been directly demonstrated in the colon of rats (7). Although this phenomenon has not been directly demonstrated in humans, human feces contain bacteria known to be able to consume H_2 , such as methane-producing (8) and sulfate-reducing (9) bacteria. If such consumption is appreciable in the colon, H_2 excretion reflects the "net" of absolute H_2 production minus H_2 consumption, and all previous in vivo and in vitro studies have measured net rather than absolute H_2 production.

The conventional method to measure the simultaneous production and consumption of a metabolite utilizes different isotopes to trace the two reactions. However, in a previous study we found that rapid exchange between the isotopes in H_2 and water precluded the use of this methodology (10). In this paper we describe a novel technique involving incubation at very low H_2 tension (PH_2)¹ that appears to provide the first independent assessment of absolute H_2 production and consumption by feces. Application of this technique demonstrated that H_2 is efficiently consumed by human feces, and that this consumption rate is enhanced by the presence of a high PH_2 and methanogenesis.

Methods

Fecal homogenates. Freshly passed feces were obtained from 11 healthy volunteers. All subjects were on an unrestricted diet and had not received antibiotics during the preceding month. The feces of seven of these subjects produced copious methane (CH_4), whereas feces from the other four produced little or no CH_4 .

A weighed sample of feces was placed in a blender vessel fitted with a gas-tight lid. After exhaustively flushing the vessel with argon, deoxygenated buffer (isotonic saline containing 0.02 M PO_4 , pH 7.2) was added. The feces were then homogenized for the minimal period (seconds) required to produce a smooth homogenate and aliquots were anaerobically aspirated into gas-tight syringes fitted with stopcocks. All syringes and flasks used in the incubations were flushed with argon before use.

1. Abbreviation used in this paper: PH_2 , hydrogen tension.

To determine if human feces were able to consume H_2 and if such consumption was influenced by P_{H_2} , 5-ml aliquots of homogenate (1:20 wt/vol) were incubated in 50-ml syringes. Gas mixtures (25 ml) composed of H_2 (concentrations of 50%, 10%, 1%, 0.1%, or 0.01%), 10% CO_2 and remainder argon were added to the syringes. Feces of six subjects (three CH_4 producers, three CH_4 nonproducers) were studied in duplicate. 0.5-ml gas samples were removed for analysis before and after 3 h of incubation. Based on previous results, this time period was selected to limit the maximal consumption to 70% of the initial H_2 so as to prevent precipitous falls in P_{H_2} that would dramatically limit additional consumption. Studies were also carried out at an initial P_{H_2} of 10% with aliquots of the homogenates that had been autoclaved before incubation.

To study the influence of P_{H_2} on net H_2 production, eight fecal samples (four CH_4 producers, four CH_4 nonproducers) were studied. A wide range of P_{H_2} in the fecal samples was obtained by incubating duplicate aliquots of homogenates (1:20 wt/vol) as follows: high P_{H_2} —2.5 ml of homogenate in 5-ml syringes with no addition of gas; intermediate P_{H_2} —2.5 ml of homogenate in 5-ml syringes with 2.5 ml of gas (10% CO_2 and 90% argon); low P_{H_2} —0.1 ml of homogenate plus 0.1 ml of sterile water in 1,000-ml flasks containing 10% CO_2 and 90% argon (0.1 ml of water was the quantity required to saturate the 1,000 ml gas space and thus prevent dehydration of the homogenate). To measure H_2 consumption by the same eight fecal samples, 2.5-ml aliquots of each homogenate were incubated with 2.5 ml of gas containing 10% H_2 , 10% CO_2 , and 80% argon. In the same way, the possibility of CH_4 consumption was studied in two fecal samples during incubation with a gas space containing 10% CH_4 . During incubation carried out at 37°C, flasks were agitated on a platform shaker at 250 rpm, while syringes were rotated at 30 rpm on a wheel.

Samples from the above incubation systems were obtained for analysis at 0, 1, 2, 4, and 24 h. 15-ml samples were obtained from the flasks. The gas containing syringes were sampled by adding 0.2 ml of argon and then removing 0.2 ml of gas, a process that resulted in an 8% dilution for each analysis. The gas volume of the syringes was measured to the nearest 0.1 ml and the amount of H_2 was then calculated from this volume and the H_2 concentration. Gas production by homogenates incubated with no gas was determined using a series of syringes. At each sampling time, 2.5 ml of argon was added to a syringe. After vigorous vortexing, a 0.2-ml gas sample was obtained for analysis. Virtually all H_2 and CH_4 should have been extracted in the gas phase and this was confirmed by experiments showing that the amount of these gases found in a repeat extraction was < 2% of the first extraction. At the end of the 24-h incubation period, the pH of incubations containing 2.5 ml of homogenate was measured using a pH meter (model 245, Corning Medical, Medfield, MA). Because of the small volume (0.1 ml) of homogenate in the flasks, pH paper was used for these determinations (EM Science, Cherry Hill, NJ). Studies comparing pH measurements obtained with the pH meter and pH paper over a pH range of 5–7 demonstrated that the paper was accurate to ± 0.5 pH units.

To determine if increasing the availability of fermentable substrate influenced the relationship between H_2 production and consumption, the above study was repeated after the addition of glucose (1% final concentration). Measurements of glucose concentration in fecal homogenates were performed before and after 24 h of incubation using a glucose oxidase technique.

Influence of P_{H_2} on H_2 production by isolated bacteria. Pure cultures of *Bacteroides fragilis* (American Type Culture Collection 23745) and *Escherichia coli* (American Type Culture Collection 29522) were grown in chopped meat broth and tryptic soy broth, respectively. The incubations were carried out in unsealed vials contained in 50-ml syringes fitted with stopcocks. After being exhaustively flushed with argon, the syringes were filled with 45 ml of gas consisting either of pure argon or 1% H_2 and 99% argon (*B. fragilis*) or 10%, 1%, 0.1% H_2 in argon (*E. coli*). Inocula (0.5 ml) from the above cultures, 4.5 ml of the respective broths, and 1 ml of 5% glucose solution were anaerobically injected through the stopcocks into the vials and incubation was

carried out at 37°C for 24 h. Gas samples (1 ml) were removed at the beginning and end of the incubation for H_2 analysis.

Breath H_2 measurements. To compare breath H_2 excretion with the in vitro H_2 production of fecal homogenates, the eight individuals who provided feces for the P_{H_2} study underwent breath H_2 testing. Breath H_2 concentration was measured in end-alveolar samples collected hourly for 8 h after the ingestion of 20 g of lactulose. Subjects fasted for 12 h before the test and during the test period. In addition, to identify low excretors of H_2 , we screened 35 healthy subjects by obtaining alveolar breath samples before and at hourly intervals for 8 h after ingestion of 10 g of lactulose. Four subjects failed to increase their breath H_2 concentration by > 20 ppm after lactulose ingestion, and thus were considered to be low H_2 excretors (11). The peak increase in breath H_2 concentration for the other 31 subjects averaged 41 ± 3 ppm. Two of the four subjects excreted negligible breath CH_4 whereas two excreted very large quantities of CH_4 (breath CH_4 concentration of these two subjects averaged 45 ppm while the average for the other 16 CH_4 excretors was 15 ± 2.6 ppm). Fecal samples were obtained from the four low excretors for measurements of H_2 production and H_2 consumption, as described above.

Gas analysis. Analyses of gas samples for H_2 and CH_4 were performed within six hours of collection by gas chromatography using a molecular sieve column and a reduction detector (Trace Analytical, Menlo Park, CA) for H_2 , and a flame ionization detector for CH_4 . The accuracy of the H_2 measurement for samples not requiring dilution (< 40 ppm) was $\pm 3\%$ and about $\pm 6\%$ for samples requiring dilution.

Sulfide measurements. It has been reported that fecal bacteria consume H_2 via reduction of sulfate to sulfide (12). To exclude the possibility that the maximal P_{H_2} achieved in the low P_{H_2} system could support such consumption, we measured fecal sulfide concentrations in the homogenates containing glucose. After 24 h, 2.25 ml of solution of zinc acetate (1.1%) was anaerobically added to the flask to prevent oxidation of sulfide. The resulting solution was collected for sulfide measurement using a modification of the method described by Cline (13) for analysis of water. Briefly, the sample was divided in three aliquots (0.6 ml). One aliquot was spiked with 10.9 μ l of sodium sulfide standard (2.6 mM) to evaluate recovery; one aliquot was treated with 48 μ l of 50% HCl and then vigorously stirred for 30 min to drive off all sulfide, the third aliquots was used for the determination of sulfide content of the specimen. The colorimetric reaction was carried out in 1.5-ml tubes that were immediately sealed after the addition of 48 μ l of diamine-ferrocyanide reagent made up in 50% HCl (13). After 30 min at room temperature samples were centrifuged at 12,000 g for 3 min and the absorbance of the supernatant was spectrophotometrically determined at 670 nm. Percentage recovery of sulfide from spiked aliquots averaged 90%. Sulfide 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 of the spiked sample.

Calculations. As will be demonstrated, human feces rapidly consumed H_2 . Therefore, observed H_2 production will be referred to as net H_2 production as opposed to the true or absolute rate of H_2 production. Net H_2 production and CH_4 production (no CH_4 consumption was observed) were calculated from the volume of these gases present at a given time point plus the volume calculated to have been previously removed for analysis. The net consumption of H_2 over a given time period was calculated from the volume of H_2 that disappeared per hour. This value was then normalized for P_{H_2} , in that H_2 consumption was shown to be directly proportional to P_{H_2} , and data were expressed as $ml \cdot h^{-1} \cdot g^{-1} \cdot atm \cdot P_{H_2}^{-1}$. The P_{H_2} was assumed to equal the arithmetic mean of the tensions present at the beginning and the end of the time period. Calculation of H_2 consumed in the production of CH_4 or sulfide was based on the ratio of 4 mol H_2 :1 mol CH_4 (8) or 1 mol sulfide (12).

The quantity of H_2 excreted in breath over 8 h was estimated from the hourly measurements of end-alveolar breath H_2 concentration and an alveolar ventilation of 4,500 ml/min. We roughly estimated the

breath H_2 excretion expected if intracolonic H_2 production was equivalent to that observed in the *in vitro* fecal incubation system. In this calculation we assumed a fecal mass of 500 g and, in accordance with Christl et al. (6) that 65% of net H_2 production was absorbed in the fasting state and 20% during the period of rapid H_2 formation after lactulose administration.

Results

The ability of human feces to consume H_2 was clearly demonstrated when homogenates were incubated with 100,000 ppm of H_2 and the gas space was sampled at intervals during a 24-h incubation (Fig. 1). The H_2 concentration above the homogenates decreased by an average of 99% over the 24-h incubation. Methanogenic feces consumed H_2 much more rapidly than did CH_4 nonproducing feces as evidenced by the significantly lower PH_2 observed at each time point ($P < 0.01$ at 1, 2, and 4 h, $P < 0.05$ at 24 h). The lower PH_2 found at 24 h in CH_4 -producing homogenates (82 ± 17 vs. $2,400 \pm 620$ ppm) suggested that H_2 consumption via CH_4 production occurs at a lower PH_2 than via other H_2 -utilizing reactions. No H_2 consumption was observed with homogenates autoclaved before incubation.

Incubation of aliquots of fecal homogenates at varying H_2 concentrations for 3 h similarly showed that homogenates that made CH_4 had a greater net consumption of H_2 (Fig. 2). For CH_4 -producing feces, the percentage of the initial H_2 that disappeared during the incubation was $29 \pm 6\%$ at an initial PH_2 of 50% and this percent consumption then increased to relatively constant values of $69 \pm 4\%$, $73 \pm 6\%$, and $63 \pm 8\%$ at initial H_2 tensions of 10%, 1.0%, and 0.10%, respectively. However, at 0.010%, H_2 consumption was not observed; rather there was a net production of H_2 that resulted in a 2.8-fold increase in the quantity of this gas relative to that initially present in the syringe. These data suggested that H_2 consumption was partially saturated at a PH_2 of 50%, but below 10%, the ml of H_2 consumed linearly declined with PH_2 , thus maintaining H_2 consumption at a constant percentage. However, at an initial PH_2 of 0.01%, consumption rate dropped below the absolute production rate and net H_2 production was observed. For fecal

Figure 1. Hydrogen consumption by human fecal homogenates. The decline in H_2 concentration in the gas space during 24 h of incubation is shown for four CH_4 -producing samples (●), four CH_4 -nonproducing samples (○), and for all eight samples (heavy line). The dotted line shows the lack of H_2 consumption by three autoclaved fecal homogenates.

Figure 2. Influence of PH_2 on net H_2 consumption by human fecal homogenates. The bars on the left show the net H_2 production observed for homogenates incubated without additional H_2 . For studies carried out with added H_2 , the dashed line shows the quantity of H_2 initially present in the syringe and the solid lines indicate the volume of H_2 remaining after 3 h of incubation for CH_4 -producing (●) and CH_4 -nonproducing (○) homogenates. The difference between the initial and observed volumes of H_2 equals net H_2 consumption or net H_2 production by the homogenates. The dotted lines represent extrapolations of the linear portions of the data (where the influence of H_2 production is negligible) to a PH_2 of 0.002%. The difference between the initial volume of H_2 and that predicted to remain after three hours of incubation at a PH_2 of 0.002% indicates that absolute H_2 consumption should be only ~ 0.00035 ml/3 h and 0.00015 ml/3 h for CH_4 producing and CH_4 nonproducing homogenates, respectively. These values would be only a small fraction of the observed H_2 production.

homogenates that did not produce CH_4 , the percentages of the initial H_2 consumed were 5.3%, 26%, and 39% at initial H_2 tensions of 50%, 10%, and 1.0%, respectively, while at 0.1% and 0.01%, 15% and 11-fold increases in H_2 were, respectively, observed. Thus, there appeared to be complete saturation of H_2 consumption at a PH_2 of 50% and partial saturation at 10%. The 44% consumption at 1% PH_2 presumably represents the maximal rate of H_2 catabolism by these fecal samples; however, this rate was not observed at lower initial H_2 tensions because absolute H_2 production exceeded the slower consumption at low PH_2 . Extrapolation of the H_2 consumption data obtained at high PH_2 to a H_2 tension of 0.002% indicates that at this low PH_2 , consumption would be only a small fraction of the observed H_2 production rate by either type of fecal sample (Fig. 2). Consumption of H_2 observed with the six autoclaved homogenates was not significantly different from zero ($1.1 \pm 1.6\%$, data not shown).

The relation observed between H_2 consumption and H_2 tension indicates that comparative analysis of the efficiency of H_2 consumption requires normalization for PH_2 . Table I shows the data presented in Fig. 1 expressed as H_2 consumption rate per atmosphere of H_2 . Consumption rates measured at 1, 2, and 4 h were significantly greater ($P < 0.03$) for CH_4 -producing feces. The fall-off in observed H_2 consumption between 4 and 24 h presumably represents the increased contribution of H_2 production to the total quantity of H_2 remaining in the system.

Fig. 3 (left) shows that PH_2 had an important effect on the net H_2 production by fecal homogenates incubated with no

Table I. Net Hydrogen Consumption by CH₄-Producing and CH₄-nonproducing Fecal Homogenates

Fecal homogenates	Incubation time	H ₂ Consumed
		ml · h ⁻¹ · g ⁻¹ · atm PH ₂ ⁻¹
CH ₄ producing (n = 4)	0-1	26±4.6*
	1-2	28±3.8‡
	2-4	16±0.55§
	4-24	0.82±0.14
CH ₄ nonproducing (n = 4)	0-1	7.8±3.2*
	1-2	7.9±3.9‡
	2-4	5.5±0.95§
	2-24	1.2±0.50

Data represent mean±SEM.

* *P* < 0.03.

‡ *P* < 0.02.

§ *P* < 0.0001.

added substrate. Aliquots of the same homogenate incubated at high, intermediate, and low PH₂ for 24 h had average net H₂ productions of 0.0008±0.0002, 0.051±0.020, and 0.67±0.12 ml H₂/g, respectively. At high and intermediate PH₂, net H₂ production peaked at 1 h (0.0059±0.0023 ml/g) and 2 h (0.087±0.0022 ml/g), respectively, and then declined at each subsequent time point. In contrast, homogenates maintained at low PH₂ showed a continuous increase in net H₂ production. Since the pH of all the homogenates was similar (never less than pH 6.5) differences in H₂ production cannot be attributed to the influence of acidity on bacterial metabolism.

The data shown in Fig. 3 were analyzed on the basis of CH₄-producing status of the homogenates. Both at high and intermediate PH₂, feces that produced CH₄ had a much lower net H₂ production than did the CH₄ nonproducing feces (Fig. 4), whereas at low PH₂, net H₂ production was similar for the two groups.

The relationship between H₂ and CH₄ production rates in the four CH₄ producing samples is shown in Table II. The low

net H₂ production observed at high and intermediate PH₂ was associated with high CH₄ formation, whereas the high H₂ production found at low PH₂ was associated with almost no CH₄ formation. The possibility that methanogenic bacteria were not viable in the low PH₂ system was excluded by the appearance of copious CH₄ when the PH₂ in the flasks was raised to 10% by addition of exogenous H₂. To determine the PH₂ in the gas space that resulted in detectable CH₄ formation, aliquots of a CH₄-producing homogenate were incubated in flasks containing H₂ tensions of 0, 10, 50, 100, 500, and 1,000 ppm. At 24 h of incubation, CH₄ was observed at 50 ppm PH₂, with increasing rates of production at higher H₂ tensions.

Supplying glucose to the homogenates markedly increased net H₂ production at each PH₂ (Fig. 3, right). The difference in net H₂ production between CH₄ producers and CH₄ nonproducers at high and intermediate PH₂ (Fig. 4, right) was even greater than observed with no added substrate. At low PH₂, there was appreciable CH₄ formation (Table II) and net H₂ production was significantly lower (*P* < 0.002) for CH₄-producing feces. However, in CH₄ producers the sum of H₂ consumed as CH₄ (3.8 ml) plus net H₂ production (9.3 ml) was similar to the net H₂ (14 ml) of CH₄ nonproducers at 24 h. Glucose concentration in the homogenates fell from 1 g/dl at the beginning of the incubation to an average of 0.09 g/dl after 24 h, indicating that 91% of the glucose was catabolized. The calculated absolute H₂ production/g of glucose fermented averaged ~ 80 ml/g.

The mean sulfide concentration in the homogenates after 24 h of incubation at low PH₂ was 6.0±0.88 μM, a value that corresponds to a sulfide content/0.1 ml of homogenate of 0.6 nmol. Given that 4 mol of H₂ are oxidized in the reduction of 1 mol of sulfate to sulfide, 0.054 μl of H₂, a negligible quantity, would have been consumed in the production of the above sulfide concentration.

Estimated breath H₂ excretion for the 8-hour period after lactulose ingestion averaged 155±12 ml for the four CH₄ producers and 226±110 ml for the nonproducers.

Culturing *B. fragilis* and *E. coli* with high initial concentrations of H₂ had little, if any, effect on H₂ production rates of these organisms (Table III). Thus, the lower H₂ production observed in fecal homogenates incubated at high PH₂ appar-

Figure 3. Influence of PH₂ on net H₂ production of four CH₄-producing and CH₄-nonproducing homogenates incubated at high (■), intermediate (Δ), and low (□) PH₂ without addition of substrate (left) and with the addition of 1% glucose (right).

Figure 4. Influence of CH₄-producing status on net H₂ production at high, intermediate, and low P_{H₂}. Data are shown for four CH₄-producing (●) and four CH₄ nonproducing (○) fecal homogenates incubated without (left) and with (right) addition of 1% glucose.

ently is not attributable to an inhibitory effect of P_{H₂} on absolute H₂ production.

The results of experiments carried out with feces from the four low H₂ excretors are summarized in Table IV. The results observed with the two fecal samples that produced CH₄ and the two that did not produce CH₄ were, respectively, compared to

Table II. Relationship between Net H₂ Production and CH₄ Formation by CH₄-producing Fecal Homogenates

Glucose addition	P _{H₂}	Net H ₂	CH ₄
		ml/g feces	
–	High	0.00030±0.00010	0.54±0.025
–	Intermediate	0.0027±0.0005	0.53±0.061
–	Low	0.70±0.25	ND (<0.005)
+	High	0.0038±0.00051	2.2±1.0
+	Intermediate	0.13±0.063	2.5±1.0
+	Low	9.3±0.69	0.94±0.77

Data represent mean±SEM at 24 h of incubation.

Table III. Influence of High P_{H₂} on Hydrogen Production by Pure Cultures of Bacteria Incubated for 24 h

Bacterial culture	Initial P _{H₂}	H ₂ produced*
	ppm	ml/24 h
<i>Bacteroides fragilis</i>	0	0.16±0.00029
<i>Bacteroides fragilis</i>	10,000	0.15±0.043
<i>Escherichia coli</i>	0	2.7±0.42
<i>Escherichia coli</i>	1,000	2.8±0.070
<i>Escherichia coli</i>	10,000	2.6±0.14
<i>Escherichia coli</i>	100,000	2.4±0.42

Data represent mean±SEM.

* Difference between initial H₂ and H₂ present at 24 h.

the control values shown in Figs. 2 and 4. Net H₂ production measurements determined in the intermediate H₂ tension system were only slightly reduced for the CH₄ nonproducing samples but were extremely low for the CH₄-producing specimens. In contrast, the absolute H₂ production of all four samples, measured in the low H₂ tension system, was similar to that of the controls. The consumption rate of H₂ by the CH₄-nonproducing feces was comparable to the control values, but was appreciably higher than that of the controls for the CH₄-producing samples.

Discussion

Information on the influence of various factors on intracolonic bacterial H₂ metabolism primarily has been obtained from in vitro studies of fecal homogenates (14–16). In all such studies H₂ production was assumed to equal the volume of H₂ recovered from the gas space of the incubation vessel. However, in addition to H₂-producing bacteria, the colon contains bacteria

Table IV. Comparison of Absolute and Apparent H₂ Production during Glucose Fermentation and H₂ Consumption of Feces from Controls and Four Subjects with a Low Breath H₂ Response to Lactulose

Type of feces	H ₂ production		H ₂ consumption
	Intermed. P _{H₂}	Low P _{H₂}	
	ml · 4h ⁻¹ · g ⁻¹		ml · 3h ⁻¹ · g ⁻¹ · atm P _{H₂} ⁻¹
CH ₄ nonproducers			
Controls*	4.9±0.33	7.3±0.15	30±3.7
Low H ₂ excretors			
Subject A	3.1	7.0	28
Subject B	3.3	7.7	36
CH ₄ producers			
Controls*	0.49±0.33	2.7±1.6	104±6.0
Low H ₂ excretors			
Subject C	0.0046	2.5	153
Subject D	0.0077	2.3	139

* Control values for H₂ consumption obtained from data shown in Fig. 2, and control values for H₂ production from data shown in Fig. 4.

that are capable of oxidizing (or consuming) H_2 . To the extent that this consumption is rapid relative to production, all previous measurements have assessed net, rather than absolute H_2 production rate.

The present study demonstrated that at high PH_2 , human feces are able to oxidize H_2 at an extremely rapid rate. For example, when the initial gas space H_2 concentration was 10% (a value commonly observed in flatus [17]), the mean H_2 consumption rate by fecal homogenates averaged 1.1 ml/h · g feces. Thus, a colonic fecal content of 500 g could consume H_2 at a rate of 550 ml/h, a value greater than any H_2 excretion rate ever reported. This rapid consumption presumably resulted from bacterial metabolism since autoclaved homogenates did not consume H_2 .

Studies of sludge have shown that sulfate reducing bacteria can outcompete methanogens for H_2 (18, 19), and it has been suggested that this relationship also exists in human feces (20). However, as shown in Fig. 1 and Table I, H_2 consumption rate was much more rapid in the CH_4 -producing group of fecal specimens as compared to those that did not produce CH_4 . For both groups these rates were relatively constant for the first 2 h of incubation. The subsequent apparent decline in H_2 consumption (despite normalization for PH_2) presumably reflects the increasing contribution of H_2 production at the low PH_2 achieved after several hours of incubation.

When studies were carried out over a wide range of initial PH_2 (from 50% to 0.01%), H_2 consumption appeared to be partially saturated at the highest concentrations but then fell in proportion to initial PH_2 over the upper part of the range (Fig. 2). However, at a low PH_2 no consumption was observed and the quantity of H_2 in the homogenate actually rose. Since absolute H_2 production presumably is constant, this increase in H_2 observed at low initial PH_2 is attributable to the inability of bacteria, at low PH_2 , to consume this gas as rapidly as it is produced. Based on the data shown in Fig. 2, we postulated that if incubation were carried out at very low PH_2 (< 0.002%), consumption should be negligible relative to production, thus permitting measurement of absolute H_2 production.

To test this hypothesis, we incubated aliquots of fecal homogenates under conditions that resulted in a wide range of H_2 tensions. An extremely low PH_2 was achieved by incubating 0.1 ml of homogenate as a thin layer at the bottom of a shaking, 1,000-ml flask. The thin layer allowed rapid equilibration of H_2 in the homogenate with the gas space. In this system, given the H_2 solubility in gas/water of 50:1, and the relative volume of gas:homogenate water of 10,000:1, virtually all H_2 will be in the gas phase. A very high PH_2 was obtained by incubating 2.5 ml of homogenate in a syringe without addition of gas, while an intermediate PH_2 was obtained by incubating 2.5 ml of homogenate with a 2.5-ml gas space. If H_2 in the liquid and the gas phase of these three systems were in equilibrium, the ratio of H_2 tensions in the homogenates would be about $1:2 \times 10^{-2}:2 \times 10^{-6}$ for equivalent net H_2 production/ml homogenate. However, in some experiments, gas production in the high and intermediate systems increased the gas space, narrowing the above ratio for expected H_2 tensions.

Studies carried out varying PH_2 clearly demonstrated the critical role of PH_2 on net H_2 production. Feces incubated without addition of substrate for 24 h at low PH_2 had an average net H_2 production 14 times and 900 times greater than observed with homogenates maintained at intermediate and high PH_2 ,

respectively (Fig. 3, *left*). At intermediate and high PH_2 , net H_2 production of CH_4 -producing feces was only a small fraction of that of CH_4 nonproducing feces (Fig. 4). This difference could reflect the more rapid H_2 consumption rate observed with CH_4 -producing feces, a concept supported by the similar net H_2 productions found at low PH_2 (Fig. 4, *left*), where CH_4 formation was negligible.

The most direct evidence that H_2 consumption was negligible at low PH_2 would be the demonstration that the metabolic products of H_2 consumption did not accumulate in the system. The two major metabolic reactions by which bacteria consume H_2 are thought to be the reduction of CO_2 to CH_4 (8) and of sulfate to sulfide (9). The production of both these metabolites was negligible when fecal homogenates were incubated at very low PH_2 . We conclude that, if a low PH_2 can be maintained, H_2 consumption is eliminated and therefore observed H_2 production equals absolute H_2 production. This absolute H_2 production was similar for CH_4 -producing and CH_4 -nonproducing feces, in contrast to the enormous differences observed in conventional incubation systems (that allow PH_2 to rise).

Feces incubated without additional fermentable substrate had an appreciable absolute H_2 production (0.67 ± 0.12 ml · g feces⁻¹ · 24 h⁻¹) indicating that fermentable material still is available at the rectum. It is not clear if this substrate is slowly metabolized dietary material or endogenous mucoproteins that have been shown to support H_2 production (3). Breath H_2 concentration expected from the above absolute production rate would be roughly 40 ppm. Since breath H_2 concentration in healthy subjects after a prolonged fast is only about 3 ppm (21), it follows that the bulk of H_2 produced in the colon during fasting is consumed and not available for excretion. The lower fasting breath H_2 concentration reported for CH_4 -producing subjects (22) can be explained by the more efficient H_2 consuming ability of methanogenic bacteria.

The addition of a rapidly fermentable substrate (glucose) markedly increased the rate of net H_2 production in all three incubation systems. In contrast to the constant gas volumes observed without added substrate, the gas spaces expanded when glucose was added to the high and intermediate systems. Therefore, the range of H_2 tensions in the three systems was diminished, an effect that should have minimized differences in H_2 consumption and net H_2 production. This hypothesis was confirmed by the finding of a ratio of 1:1.4:3.7 for net H_2 production over 24 h in the high/intermediate/low PH_2 systems compared to a ratio of 1:64:900 when no substrate was added (Fig. 3).

As in the experiments with no added substrate, both at high and intermediate PH_2 , CH_4 -producing feces had a much reduced net H_2 formation (Fig. 4, *right*). Owing to the added substrate, PH_2 in the low PH_2 system rose to a level of about 50 ppm, a value that allows H_2 consumption via CH_4 formation. In this system, the sum of the averages of net H_2 production (9.3 ml/g feces · 24 h) and H_2 consumed as CH_4 (3.8 ml/g feces · 24 h) yielded a value similar to the H_2 production observed with CH_4 nonproducing feces (14 ml/g feces · 24 h). This latter value appears to reflect absolute H_2 production since sulfide, the major metabolite of H_2 consumption of nonmethanogenic feces, did not accumulate during the incubation.

A high PH_2 could reduce net H_2 production by enhancing consumption (as demonstrated above) and/or inhibiting H_2 production. It is known that H_2 liberation by certain bacteria

(e.g., *Diplococcus glycinophilus* [23]) is inhibited by a PH_2 of 25%, while other organisms (e.g. *Veillonella gazogenes* [24]) maintain the ability to produce H_2 under one atmosphere of H_2 . We are not aware of similar data for bacteria indigenous to the human intestine. Our results (Table III) showed that high H_2 tensions had little, if any influence on H_2 liberation by pure cultures of two typical colonic bacteria (*B. fragilis*, *E. coli*). While an effect on H_2 production cannot be totally excluded, it seems likely that the major effect of PH_2 is on the rate of H_2 consumption.

From our study it is apparent that PH_2 and the ability of colonic bacteria to produce CH_4 should be major determinants of net H_2 production. For a given rate of H_2 production, both in the colonic lumen and in our in vitro fecal incubation systems, fecal PH_2 will be a function of the efficiency of fecal stirring and the gas volume to which feces are exposed. Stirring permits the rapid movement of H_2 from feces to the surrounding gas space, a process that would be very slow if H_2 had to reach the gas space solely by diffusion. Because of the high solubility of H_2 in gas compared to water, equilibration with a relatively small volume of gas produces a dramatic fall in PH_2 in the fecal material.

Our results demonstrate that, despite comparable rates of H_2 production, the amount of H_2 released from well-stirred fecal contents would be many-fold greater than from poorly stirred feces. While there are no quantitative data on stirring of colonic contents, it is tempting to speculate that efficient colonic mixing explains why some subjects have symptoms of excessive gas such as abdominal distention and flatulence in spite of delivery of normal quantities of fermentable substrate to the colon. The marked day-to-day variations in an individual's breath H_2 response to a given dose of non-absorbable carbohydrate and the sudden increase in H_2 excretion reported during periods of stress (25) more likely are attributable to variations in colonic stirring than to acute alterations in the colonic flora.

If PH_2 is allowed to rise, net H_2 production of CH_4 -producing feces is only a trivial fraction of that of CH_4 nonproducing feces. After 24 h of incubation with glucose, the ratio of net H_2 production between the two groups was 1:67 and 1:1,700 for the intermediate and high PH_2 systems, respectively. Since breath H_2 excretion reflects net H_2 production, one might expect that carbohydrate malabsorption would cause only a trivial rise in breath H_2 in CH_4 -producing relative to CH_4 -nonproducing subjects. However, breath H_2 excretion after lactulose ingestion was only 32% less in our CH_4 producers, a result that agrees with a previous study in larger groups of subjects (22). Two possible explanations for this higher than predicted H_2 excretion are that fecal PH_2 is maintained at an extremely low level, or that the production of H_2 and CH_4 is occurring in different locations in the colon. Comparison of in vitro net H_2 production by feces of CH_4 -producing subjects with their breath H_2 excretion indicated that breath H_2 excretion after lactulose was 99 times greater than predicted from carbohydrate fermentation in the intermediate PH_2 system, but comparable to that predicted from the low PH_2 system, in which the maximal PH_2 was 55 ppm. Since flatus H_2 concentration seldom is < 1,000 ppm (1) the first explanation can be excluded. Thus, we conclude that the site of lactulose fermentation is physically separated from that of CH_4 formation, a concept supported by studies in CH_4 producers showing that CH_4 pro-

duction occurs mainly in the left colon (26, 27) while the right colon should be the primary site of lactulose fermentation.

As the predominant site of fermentation moves from the right to the left colon, major differences in net H_2 production should be expected. The decreasing liquidity of feces that occurs during passage through the colon limits the efficiency of colonic mixing. As a consequence, fecal PH_2 and H_2 consumption rate will rise. In addition, a methanogenic flora, if present, is primarily localized to the left colon (27). Exposure of H_2 to methanogens results in very rapid H_2 consumption, particularly when the PH_2 is high due to inefficient stirring. Thus, slowly fermentable substances that are metabolized along the extent of the colon might be expected to yield far less net H_2 per gram than substrates that are completely fermented in the right colon. The finding that H_2 excretion was far less than expected following malabsorption of slowly, but completely, fermented starches (green banana, cold potato) (28) lends credence to this concept.

The inability of many individuals to elevate their breath H_2 excretion despite documented carbohydrate malabsorption (29, 30) limits the applicability and interpretation of H_2 breath tests. This phenomenon has been attributed to a lack of a H_2 producing flora. However, a wide variety of colonic bacteria are able to liberate H_2 , and the inability to excrete H_2 often is transitory, in contrast to the remarkable stability of the composition of the colonic flora (31). It seems possible that enhanced H_2 consumption, as opposed to decreased absolute production, could explain this apparent lack of H_2 production. Such increased consumption could result from decreased fecal stirring, an increase in numbers and/or efficiency of H_2 consuming fecal organisms, or a migration of methanogens from the left to the right colon. Application of the techniques described in this paper made it possible partially to elucidate the origin of an unusually low H_2 excretion of four subjects who failed to increase their breath H_2 concentration by > 20 ppm after ingestion of lactulose (the commonly employed criterion for diagnosing carbohydrate malabsorption [11]). When incubated in the low PH_2 system, fecal samples from all four subjects liberated H_2 at a rate comparable to that of the controls (Table IV). Thus, the feces of so-called " H_2 nonproducers" are capable of producing H_2 at a normal rate, and the reduced H_2 excretion of these subjects apparently must reflect excessively rapid H_2 consumption. Since feces from the two CH_4 nonproducers consumed H_2 at a normal rate, an in vivo phenomenon not reflected by the fecal homogenates, e.g., poor luminal stirring, presumably accounts for the elevated H_2 consumption of these subjects. In contrast, feces from the other two low H_2 excretors had very rapid in vitro consumption of H_2 and a high production rate of CH_4 . These two individuals had the highest breath CH_4 concentrations observed in 35 subjects. Thus, the low H_2 excretion of these subjects is apparently attributable to the efficient H_2 consumption of their methanogenic flora, although additional abnormalities of luminal stirring or right colonic migration of methanogens cannot be excluded.

A proposed stoichiometry for fermentation in the colon suggests that the metabolism of 1 g of glucose by fecal bacteria should liberate ~ 340 ml of H_2 (4). However, our measurements of absolute H_2 production during glucose fermentation averaged ~ 80 ml/g. Thus, it seems likely that fermentation by fecal bacteria involves some metabolic pathways that do not liberate H_2 . We conclude that people are spared from the enor-

mous gaseous distension that would result from the above stoichiometry because the absolute H₂ production is lower than predicted, and this absolute production rate is further reduced by bacterial consumption.

Excessive flatulence commonly has been considered to be simply a manifestation of the delivery of excessive carbohydrate to the colonic bacteria and therapy has been solely directed toward limiting carbohydrate malabsorption. The present study demonstrates the extraordinary importance of H₂ consumption on intestinal gas accumulation and suggest that manipulations that alter luminal stirring and/or the H₂ consuming flora could represent new therapeutic approaches to flatulence.

References

1. Levitt, M. D., and J. H. Bond. 1970. Volume, composition and source of intestinal gas. *Gastroenterology*. 59:921-929.
2. Levitt, M. D. 1969. Production and excretion of hydrogen gas in man. *N. Engl. J. Med.* 281:122-127.
3. Perman, J., and S. Modler. 1982. Glycoproteins as substrates for production of hydrogen and methane by colonic bacterial flora. *Gastroenterology*. 83:388-393.
4. Weaver, G. A., J. A. Krause, T. L. Miller, and M. J. Wolin. 1989. Constancy of glucose and starch fermentations by two different human faecal microbial communities. *Gut*. 30:19-25.
5. Grimble, G. 1989. Fibre, fermentation, flora, and flatus. *Gut*. 30:6-13.
6. Christl, S. U., P. R. Murgatroyd, G. R. Gibson, and J. H. Cummings. 1990. Quantitative measurement of hydrogen and methane from fermentation using a whole body calorimeter. *Gastroenterology*. 98:A164. (Abstr.)
7. Levitt, M. D., T. Berggren, J. Hastings, and J. H. Bond. 1974. Hydrogen (H₂) catabolism in the colon of the rat. *J. Lab. Clin. Med.* 84:163-167.
8. Smith, C. J., and M. P. Bryant. 1979. Introduction to metabolic activities of intestinal bacteria. *Am. J. Clin. Nutr.* 32:149-157.
9. Gibson, G. R., G. T. Macfarlane, and J. H. Cummings. 1988. Occurrence of sulphate-reducing bacteria in human faeces and the relationship of dissimilatory sulphate reduction to methanogenesis in the large gut. *J. Appl. Bacteriol.* 65:103-111.
10. Strocchi, A., P. D. Klein, W. Wong, and M. D. Levitt. 1992. Use of deuterium to study H₂ consumption by human feces: evidence for rapid deuterium-hydrogen exchange. *Biochem. Arch.* 8:33-38.
11. Newcomer, A. D., D. B. McGill, P. J. Thomas, and A. F. Hofmann. 1975. Prospective comparison of indirect methods for detecting lactase deficiency. *N. Engl. J. Med.* 293:1232-1236.
12. Gibson, G. R., J. H. Cummings, G. T. Macfarlane, C. Allison, I. Segal, H. H. Vorster, and A. R. P. Walker. 1990. Alternative pathways for hydrogen disposal during fermentation in the human colon. *Gut*. 31:679-683.
13. Cline, J. D. 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnol. Oceanogr.* 14:454-458.
14. Bond, J. H., and M. D. Levitt. 1972. Use of pulmonary hydrogen (H₂) measurements to quantitate carbohydrate malabsorption: study of partially gastrectomized patients. *J. Clin. Invest.* 51:1219-1225.
15. Perman, J. A., S. Modler, and A. C. Olson. 1981. Role of pH in production of hydrogen from carbohydrates by colonic bacterial flora. *J. Clin. Invest.* 67:643-650.
16. Bjørneklett, A., and E. Jenssen. 1982. Relationships between hydrogen (H₂) and methane (CH₄) production in man. *Scand. J. Gastroenterol.* 17:985-992.
17. Kirk, E. 1949. The quantity and composition of human colonic flatus. *Gastroenterology*. 12:782-794.
18. Lupton, F. S., and J. G. Zeikus. 1984. Physiological basis for sulfate-dependent hydrogen competition between sulfidogens and methanogens. *Curr. Microbiol.* 11:7-12.
19. Lovley, D. R., D. F. Dwyer, and M. J. Klug. 1982. Kinetic analysis of competition between sulfate reducers and methanogens for hydrogen in sediments. *Appl. Environ. Microbiol.* 43:1373-1379.
20. Gibson, G. R., J. H. Cummings, and G. T. Macfarlane. 1988. Competition for hydrogen between sulphate-reducing bacteria and methanogenic bacteria from the human large intestine. *J. Appl. Bacteriol.* 65:241-247.
21. Levitt, M. D., P. Hirsh, C. A. Fetzner, M. Sheahan, and A. S. Levine. 1987. H₂ excretion after ingestion of complex carbohydrates. *Gastroenterology*. 92:383-389.
22. Cloarec, D., F. Bornet, S. Gouilloud, J. L. Barry, B. Salim, and J. P. Galmiche. 1990. Breath hydrogen response to lactulose in healthy subjects: relationship to methane producing status. *Gut*. 31:300-304.
23. Barker, H. A., B. E. Volcani, and B. P. Cardon. 1948. Tracer experiments on the mechanism of glycine fermentation by *Diplococcus glycinophilus*. *J. Biol. Chem.* 173:803-804.
24. Johns, A. T. 1951. The mechanism of propionic acid formation by *Veillonella gazogenes*. *J. Gen. Microbiol.* 5:326-336.
25. Calloway, D. H., and E. L. Murphy. 1968. The use of expired air to measure intestinal gas formation. *Ann. NY Acad. Sci.* 150:82-95.
26. Levitt, M. D., and F. J. Ingelfinger. 1965. Hydrogen and methane production in man. *Ann. NY Acad. Sci.* 150:75-81.
27. Flourie, B., F. Etanchaud, C. Florent, P. Pelletier, Y. Bouhnik, and J-C Rambaud. 1990. Comparative study of hydrogen and methane production in the human colon using caecal and faecal homogenates. *Gut*. 31:684-685.
28. Cummings, J. H., and H. N. Englyst. 1990. Breath hydrogen (H₂) may not be a reliable way of quantitating starch fermentation. *Gastroenterology*. 98:A166. (Abstr.)
29. Levitt, M. D., and R. M. Donaldson. 1970. Use of respiratory hydrogen (H₂) excretion to detect carbohydrate malabsorption. *J. Lab. Clin. Med.* 75:937-945.
30. Gilat, T., H. Ben Hur, E. Gelman-Malachi, R. Terdiman, and Y. Peled. 1978. Alterations of colonic flora and their effect on the hydrogen breath test. *Gut*. 19:602-605.
31. Bornside, G. H. 1978. Stability of human fecal flora. *Am. J. Clin. Nutr.* 31:S141-S144.