

## Role of pH in production of hydrogen from carbohydrates by colonic bacterial flora. Studies in vivo and in vitro.

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### Research Article

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# Role of pH in Production of Hydrogen from Carbohydrates by Colonic Bacterial Flora

## STUDIES IN VIVO AND IN VITRO

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**ABSTRACT** Hydrogen produced by colonic bacteria and excreted in breath is a useful index of carbohydrate malabsorption. Since colonic contents are often acidic in individuals with carbohydrate malabsorption and in normal newborns, we determined the effect of colonic acidification on H<sub>2</sub> production. Acidification of colonic contents by dietary means significantly reduced excess breath H<sub>2</sub> excretion from 55.4±11.1 (SEM) to 12.2±3.1 ml/4 h (*P* < 0.05) after administration of 0.3 g/kg of the nonabsorbable sugar lactulose to five normal adult subjects. Similarly, the breath H<sub>2</sub> response to lactose was reduced or eliminated in two proven lactose malabsorbers after acidification. The correlation between pH and H<sub>2</sub> production from carbohydrate was further investigated in adults and neonates, using an in vitro fecal incubation system. Glucose disappearance and H<sub>2</sub> production were pH dependent and highly correlated (*r* = 0.94) in the pH range 5.5–7.6. Maximal production of H<sub>2</sub> from glucose by fecal incubates occurred at pH 7.0–7.45. Inhibition of H<sub>2</sub> production from carbohydrate occurred at acid pH. H<sub>2</sub> per hour from glucose at pH 6.2 and 5.5 averaged 60.2% and 24.2%, respectively, of that produced at neutral pH. Rapid reversal of pH-induced inhibition by neutralization indicated a metabolic, rather than a bactericidal process. The observations indicate that the breath H<sub>2</sub> response to malabsorbed carbohydrate is affected by colonic pH. It appears that the efficiency of bacterial carbohydrate metabolism in the colon is pH dependent.

## INTRODUCTION

Measurement of H<sub>2</sub> in breath is a useful, noninvasive method for detecting carbohydrate malabsorption

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(1–7). Dietary carbohydrate reaching the colon is metabolized by microorganisms that convert sugars to organic acids, CO<sub>2</sub>, and H<sub>2</sub>. A fraction of the gaseous products is transferred to the portal circulation and excreted in the lungs.

The effect of colonic pH on bacterial intraluminal carbohydrate metabolism and H<sub>2</sub> production has not been well defined. Gas production in vitro from bean homogenates by canine intestinal bacteria is inhibited at acidic pH (8), and Vince et al. (9) have recently demonstrated that generation of ammonia by colonic bacteria is pH dependent. Colonic pH is frequently acidic in individuals on certain modified diets (11) and in normal newborns (12). Luminal bacterial metabolism of dietary carbohydrates may be altered and H<sub>2</sub> production reduced in these subjects, which may affect the interpretation of H<sub>2</sub> breath tests used in the diagnosis of carbohydrate malabsorptive states. Furthermore, the potential application of H<sub>2</sub> measurements for noninvasive investigation of carbohydrate absorptive capacity in newborns may be affected by their frequently acidic colonic pH.

The purpose of the present study was to determine the effect on breath H<sub>2</sub> excretion of colonic acidification induced by the addition of nonabsorbed carbohydrate to the diet, and the influence of pH on H<sub>2</sub> production from carbohydrates by colonic bacteria of adults and neonates, using an in vitro fecal incubation system. The results demonstrate that consumption of carbohydrate substrates and H<sub>2</sub> production by human colonic bacteria are directly related to luminal pH.

## METHODS

### *Human subjects*

Studies were performed with five healthy adult volunteers, two of whom were lactose intolerant and had previous lactose-H<sub>2</sub> breath test results consistent with lactose malabsorption.

There were four females and one male, ranging in age from 26 to 40 yr. None excreted methane (<1 ppm CH<sub>4</sub> above room air in expired air) as determined by mass spectrometry as described below. Methane excretors were excluded because their H<sub>2</sub> response to intake of a nonabsorbable carbohydrate may be different from the non-CH<sub>4</sub> excreting population (13). All subjects remained on their accustomed diets while receiving supplements of lactulose syrup (Merrell-National Laboratories, Cincinnati, Ohio), 20–40 ml (0.3 g/kg) twice daily for 7 d (lactulose maintenance).

Fresh stool was obtained for pH measurement before and upon conclusion of lactulose maintenance. Specimens were collected into a plastic container, and the pH was immediately measured using a radiometer TTTC1 pH meter by bringing the calibrated electrode in contact with the stool. The validity of this method has been previously established (14).

*Effect of acidification on the lactulose-H<sub>2</sub> breath test.* The initial dose of lactulose (day 1) also served as a test dose, administered at 0800 h after an overnight fast. A second test dose (0.3 g/kg) was administered at 0800 h on the morning after the 7-d maintenance period (day 8) within 12 h of the final maintenance dose. Breath H<sub>2</sub> concentration was monitored before (base line) and at half-hour intervals for 4 h after administration of the test doses. Subjects did not smoke during the breath tests. Breath samples were obtained by the nasal prong technique (7), aspirating aliquots of air into 60-ml plastic syringes equipped with 3-way stopcocks. H<sub>2</sub> in the samples was analyzed within 4 h of collection. Stability of samples using this methodology has been previously established (7).

Preliminary trials indicated that the lactulose dosage did not induce urgency and liquid stools. However, daily stool weight, measured in one subject, increased from 83±35 (mean ± SEM of 3 d pre-lactulose) to 265±34 g during the lactulose maintenance period, and stool frequency increased from 1 to 1–2 stools/d occurring each morning. The influence of laxation on the H<sub>2</sub> response to lactulose in this subject was therefore investigated. A magnesium sulfate mixture, previously shown not to acidify colonic contents (15), was administered over a 3-d period, and the dosage was adjusted upward from 5 to 15 ml three times daily to produce a stool weight and frequency comparable to that observed when the same subject received lactulose. A test dose of lactulose (0.3 g/kg) was administered on the morning before beginning magnesium sulfate and again on the morning of day 3 of magnesium sulfate intake, immediately after output of a stool volume that exceeded daily output while on lactulose maintenance. This study was separated from the lactulose maintenance period by 1 mo.

*Effect of acidification on the lactose-H<sub>2</sub> breath test.* The two lactose malabsorbers received a test dose of lactose 0.3 g/kg before the 7-d lactulose maintenance period and again 12 h after an additional lactulose dose given in the evening of day 8. One of these subjects also received a test dose of lactose 0.3 g/kg 4 h after a 24-h period in which lactulose 0.4 g/kg was administered four times at 8-h intervals. Interval breath H<sub>2</sub> concentrations were determined before, and at half-hour intervals for 3 h after each lactose load. Informed consent was obtained from all subjects.

### Fecal incubations

Incubations of fecal specimens obtained from adults and neonates were performed by a modification of the method of Bond and Levitt (16). Modification was necessitated by the relatively small size of the fecal specimens from neonates.

*Effect of pH on H<sub>2</sub> production by fecal homogenates from adults.* Fresh fecal specimens (~10 g) were obtained from

five healthy non-CH<sub>4</sub> producing adult subjects ages 27–45 while they consumed their regular diets. Preliminary experiments demonstrated that homogenization of stools with phosphate-saline solutions ranging from pH 3.0 to 11.0 resulted in homogenates with initial pH values ranging from 5.0 to 9.1, respectively. Specimens were therefore divided and homogenized in three times their weight of 0.1 M potassium phosphate-buffered saline (pH 7.0), and in identical solutions in which initial pH was adjusted to 3.0, 5.0, 8.6, 10.0, and 11.0 with 1 N solutions of hydrochloric acid or sodium hydroxide. Each specimen was incubated with solutions at three or more of the specified pH levels.

Aliquots (1 ml) of each stool homogenate were placed in a series of 50-ml Vacutainers (Becton-Dickinson & Co., Rutherford, N. J.) containing 1 ml 1.25% glucose (69 μmol). The pH of each incubate was measured using a Radiometer TTTC1 pH meter (Radiometer, Copenhagen, Denmark), Vacutainers were flushed with nitrogen, and sealed with rubber stoppers to insure gas tightness. Incubations were begun within 10 min of stool collection. Incubates were prepared in quadruplicate for each homogenate. One incubate was immediately boiled (3 min) to inactivate microorganisms for subsequent determination of initial glucose content. The remaining incubates were placed in a 37°C water bath for 1 h. Control samples of stool homogenate alone and glucose alone were also incubated. The gas in one experimental incubate and the control incubates was recovered upon completion of incubation by displacement of gas with water. Incubation in the remaining two vacutainers was terminated by immersion in water at 100°C, the final pH was determined, and final glucose content measured.

*Effect of pH on H<sub>2</sub> production by fecal homogenates from neonates.* Fresh fecal specimens (~2 g) were obtained from five healthy neonates ages 2–28 d (three breast-fed and two fed lactose-containing formulas), and divided equally. Preliminary experiments demonstrated that comparable amounts of H<sub>2</sub> were produced from glucose by fecal specimens homogenized in either saline or phosphate-saline. Specimens were therefore homogenized in three times their weight of saline alone (native condition) and in three times their weight of 0.1 M phosphate-buffered saline at pH 7.0. As in the adult studies, a 1:1 ratio of stool homogenate and substrate solution was maintained. Aliquots (0.3 ml) of each stool were placed in a series of 50-ml Vacutainers containing 0.3 ml 1.25% glucose, lactose, sucrose, or lactulose (as powder from Sigma Chemical Co., St. Louis, Mo.). The actual pH of the incubates was measured, and the vacutainers were flushed with nitrogen, sealed with rubber stoppers, and incubated in a 37°C water bath for 1 h. Control samples of stool homogenate alone were also incubated. The gas in each sample at the end of 1 h was recovered as in the incubates from adults.

*Effect of neutralization of acidic incubates on H<sub>2</sub> production.* The effect of reversal of acidic conditions on H<sub>2</sub> production from glucose by fecal homogenates was determined by reincubation of 1 h incubates, prepared and analyzed as described above, for a 2nd h after addition of 1 ml of a 0.1 M phosphate-saline solution (pH 10). 2nd-h control samples were prepared by addition of 1 ml of solution of pH equal to that at the end of the 1st h incubation. Determination of H<sub>2</sub>, glucose concentrations, and pH was performed after completion of the reincubation. An additional control was prepared by incubation of a sample at neutral pH for 1 and 2 h.

*Effect of osmolality on H<sub>2</sub> production in vitro.* The osmolality of the phosphate-saline solutions ranged from 475 to 541 mosmol/kg H<sub>2</sub>O. A control experiment using a fecal specimen was therefore performed to determine the range of pre- and postincubation osmolalities after homogenization with these solutions. Osmolalities were 267, 268, 254, and 267

mosmol/kg before incubation and 260, 245, 268, and 325 mosmol/kg following 1-h incubation of a fecal specimen with solutions of pH 3.0, 5.0, 7.0, and 10.0, respectively. The effect of osmolality on H<sub>2</sub> production from glucose by colonic bacteria was determined by incubating fecal homogenates prepared as above with 55 μmol glucose in volumes of water ranging from 0.5 to 10.0 ml, which was sufficient to achieve osmolalities of incubates ranging from 50 to 450 mosmol/kg H<sub>2</sub>O.

Reproducibility of the *in vitro* method for H<sub>2</sub> production was assessed by 1-h incubation of five samples prepared from a single stool homogenate. H<sub>2</sub> measurements varied by a maximum of 4.7%.

## Analyses

H<sub>2</sub> was measured by gas chromatography or mass spectrometry. Methane was measured by mass spectrometry. The gas chromatograph (Carle Instruments, Anaheim, Calif.) was equipped with a microthermistor detector. Samples of expired air and gas produced from incubations (10 ml) were applied to a molecular sieve column at 37°C using zero-grade argon as the carrier gas. Larger samples of expired air (50 ml) were also applied to a dedicated mass spectrometer (Honeywell Multigas Analyzer, Honeywell, Inc., Minneapolis, Minn.), which was a functionally self-contained quadrupole equipped with a pulsed leak injection system and pumped with an integral ion pump. For both gas chromatography and mass spectrometry, gas concentrations were determined by comparing the peak height of the unknown with that of a H<sub>2</sub> or CH<sub>4</sub> standard (Liquid Carbonic Corp., Chicago, Ill.) of known concentration. The minimum detectable concentration of H<sub>2</sub> or CH<sub>4</sub> was 1 ppm with either instrument. Excess H<sub>2</sub> production from expired air samples was determined by integrating the area under the curve of interval breath H<sub>2</sub> concentrations (17) and expressing data as milliliters per total test period.

Osmolality was measured by freezing-point depression with an Osmette precision Osmometer (Precision Systems, Sudbury, Mass.). Glucose was determined by a glucose-oxidase method (Statzyme, Worthington Biochemical Corp., Freehold, N. J.). Boiled incubates were filtered through 0.45-mm Millex sterile disposable filters (Millipore Corp., Bedford, Mass.) to eliminate precipitates. 10-μl aliquots of filtered solutions were used in the glucose analysis. Absorbance measurements were performed on a Beckman DK-2A spectrophotometer set at 500 nm (Beckman Instruments, Fullerton, Calif.). Standards were prepared from glucose (Sigma Chemical Co., St. Louis, Mo.). Incubation with stool did not interfere with the glucose determinations, because addition of known aliquots of glucose to fecal incubates gave a correct increase in absorbance.

Statistical analyses for significance were performed using Student's paired *t* test. The dependence of H<sub>2</sub> production on osmolality was statistically evaluated by determining the equations of linear regression for points between 200 and 450 mosmol/kg H<sub>2</sub>O and testing the 95% confidence limits of the slope.

## RESULTS

### *In vivo* studies

**Effect of acidification on the lactulose-H<sub>2</sub> breath test.** Ingestion of lactulose for 1 wk by five healthy subjects reduced fecal pH from 7.1±0.3 (SEM) before to 5.8±0.6 after lactulose maintenance. Base-line H<sub>2</sub>

concentrations measured immediately before administration of lactulose test doses did not differ significantly (*P* > 0.05) before and after the lactulose maintenance period (21.4±6.7 ppm on day 1 and 16.7±5.1 ppm on day 8). In contrast, a blunted H<sub>2</sub> response to the test dose occurred over the ensuing 4 h (Fig. 1). Excess breath H<sub>2</sub> per 4 h declined significantly (*P* < 0.05) from 55.4±11.1 on day 1 to 12.2±3.1 ml on day 8 (Fig. 2). In the subject whose H<sub>2</sub> was least affected (28.8 vs. 18.5 ml/4 h), stool pH remained at 7.0 despite lactulose maintenance.

Inhibition of the breath H<sub>2</sub> response to lactulose coincided with colonic acidification in one subject who was monitored daily during the 7-d lactulose maintenance period. Fecal pH declined sharply from 6.8 on day 2 to 5.8 on day 3, and excess H<sub>2</sub> per 4 h after the morning lactulose doses fell fourfold from 30.5 to 7.3 ml over this 24-h interval. Fecal pH ranged from 5.8 to 6.0 during the balance of the maintenance period, and H<sub>2</sub> per 4 h did not exceed 13.9 ml. Omission of lactulose for 24 h upon conclusion of the maintenance period caused a rise in pH from 6.0 to 6.9, and H<sub>2</sub> per 4 h after a lactulose test dose given immediately after the 24-h lactulose-free period rose to 32.0 ml. This value closely approximated the H<sub>2</sub> response corresponding to fecal pH 6.8 on day 2 of lactulose maintenance.

Inhibition of the breath H<sub>2</sub> response to lactulose was not attributable to a laxative effect of lactulose. Administration of magnesium sulfate to one subject in amounts sufficient to increase stool output and frequency to 551 g/d and 2/d, respectively, vs. 265 g/d and 1–2/d while on lactulose maintenance, was associated with a rise in excess breath H<sub>2</sub>/4 h in response to a lactulose test dose from 17.1 ml before to 34.0 ml immediately after magnesium sulfate-induced laxation. This increase corresponded with a rise in fecal pH from 6.5 before to 7.35 following magnesium sulfate ingestion.

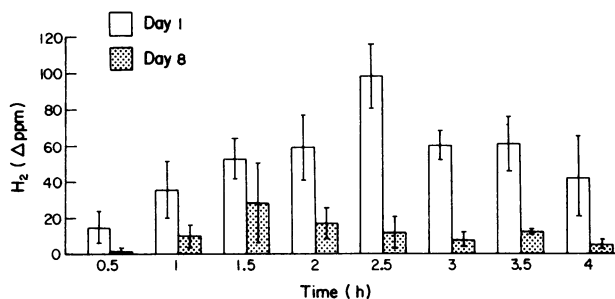


FIGURE 1 Breath H<sub>2</sub> responses to lactulose test doses on day 1 and on day 8, after the lactulose maintenance period. Samples of expired air were obtained at half-hour intervals following administration of lactulose 0.3 g/kg. The average change in H<sub>2</sub> concentration in parts per million above base-line (fasting) levels (Δppm) for five subjects is represented by the bars (mean±SEM).

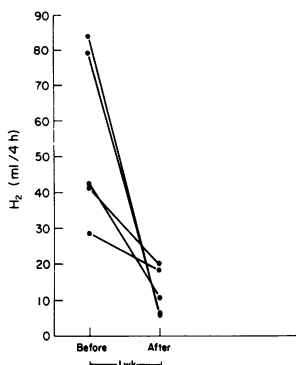


FIGURE 2 Excess  $H_2$  produced after test dose of lactulose before and after addition of lactulose to the diet. Excess  $H_2$  production for five subjects was determined by integrating the area under the curve of interval breath  $H_2$  concentrations according to the method of Solomons et al. (17).

#### Effect of acidification on the lactose $H_2$ breath test.

Two subjects had positive lactose- $H_2$  breath tests consistent with lactose malabsorption. In both, the  $H_2$  response to lactose was reduced or eliminated by lactulose ingestion for 1 wk (Fig. 3). In one lactose malabsorber, no rise in  $H_2$  occurred following a lactose test after a single 24-h period on lactulose (0.4 g/kg q 8 h) which induced 2 semi-liquid stools and reduced the stool pH to 4.3.

#### In vitro studies

**Effect of pH on  $H_2$  production by fecal homogenates from adults.** Mean stool pH in five adults, one studied twice over a 2-wk interval, was  $6.6 \pm 0.3$ . Homogenization in phosphate-saline solutions of pH 10.0 ( $n = 6$ ), 7.0 ( $n = 6$ ), 5.0 ( $n = 5$ ) and 3.0 ( $n = 3$ ) produced fecal incubates of pH  $7.6 \pm 0.15$ ,  $7.0 \pm 0.09$ ,  $6.2 \pm 0.10$ , and  $5.5 \pm 0.25$ , respectively.

$H_2$  per hour produced by fecal homogenates incubated with glucose did not differ ( $P > 0.05$ ) at pH 7.6 and 7.0 ( $16.5 \pm 3.2$  and  $16.1 \pm 2.6 \mu\text{mol}$ , respectively). In contrast, reduction of pH to 6.2 and 5.5 caused a significant decrease in  $H_2$  production to  $9.7 \pm 2.5$  and  $3.9 \pm 3.8 \mu\text{mol/h}$  ( $P < 0.05$  when compared with pH 7.0).  $H_2$  per hour produced by incubation of a fecal specimen

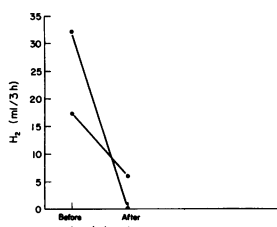


FIGURE 3 Breath  $H_2$  response to a lactose test dose (0.3 g/kg) before and after addition of lactulose to the diet in two subjects. Excess  $H_2$  was determined as in Fig. 2.

at initial pH varying from 5.5 to 9.0 indicated that the pH optimum for  $H_2$  production from glucose occurred between 7.0 and 7.45 (Fig. 4).

$H_2$  production from glucose by colonic flora varied among subjects, but was constant at neutral pH in the subject studied twice. The relationship between initial pH and  $H_2$  produced was demonstrable in all subjects. The smallest amounts of  $H_2$  were produced at the most acidic initial pH, and were associated with the smallest differences between initial and final pH of the incubate.  $H_2$  per hour produced by fecal incubates to which no substrate was added (controls) demonstrated the same relationship to pH exhibited by experimental samples:  $0.27 \pm 0.25$ ,  $0.18 \pm 0.16$ ,  $0.08 \pm 0.07$ , and  $0.02 \pm 0.02 \mu\text{mol/h}$  at pH 7.6, 7.0, 6.2, and 5.5, respectively.

$H_2$  production was highly correlated ( $r = 0.94$ ,  $y = 0.39x - 5.02$ ,  $P < 0.01$ ) with glucose consumption. The linear regression indicated that  $0.39 \mu\text{mol } H_2$  was evolved per micromole of glucose consumed.

**Effect of pH on  $H_2$  production by fecal homogenates from neonates.** The effect of pH on  $H_2$  production from glucose was reproduced with lactulose, lactose, and sucrose using fecal incubates from neonates. The mean pH of fecal specimens from five neonates was  $6.0 \pm 0.4$  when homogenized in unbuffered saline, and  $6.9 \pm 0.1$  when duplicate fecal specimens were homogenized in 0.1 M phosphate-buffered saline (pH 7.0). As shown in Table I, a greater than twofold increase in  $H_2$  production from each of the four carbohydrates occurred at pH 6.9 compared with 6.0 ( $P < 0.005$ ). Addition of glucose, lactulose, lactose, or sucrose to fecal homogenates at pH 6.9 yielded 10-fold differences in  $H_2$  per hour among samples, ranging from 1.8 to  $17.6 \mu\text{mol}$ . Expressed as percentage of  $H_2$  produced from glucose,  $H_2$  per hour produced from different substrates

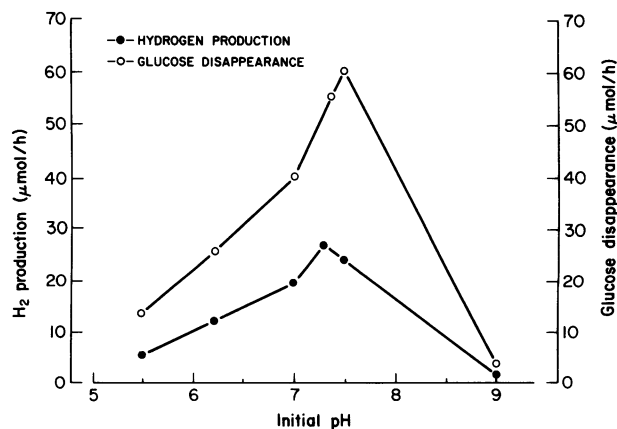


FIGURE 4 Relationship between pH and  $H_2$  production from glucose by colonic flora. A fecal specimen was divided, homogenized in a series of phosphate-saline solutions at pH 3.0–11.0, and incubated for 1 h in 1 ml 1.25% glucose ( $69 \mu\text{mol}$ ). The pH values represent the actual pH at the start of incubation.

TABLE I  
H<sub>2</sub> Production from Various Carbohydrates by  
Fecal Homogenates from Newborns

Substrate	H <sub>2</sub> production		P*
	pH 6.0±0.4	pH 6.9±0.1	
	μmol/h		
Glucose	3.5±1.5	8.2±3.4	0.001
Lactulose	3.3±1.3	7.5±3.4	0.005
Lactose	3.4±1.1	7.7±2.8	0.005
Sucrose	3.4±1.4	8.7±3.3	0.005

Fecal specimens from neonates were homogenized in either saline or phosphate-buffered saline (pH 7.0) and incubated for 1 h with each of four substrates. The mean pH (±SEM) of the incubates were 6.0±0.4 and 6.9±0.1 when specimens were homogenized in saline or phosphate-buffered saline (7.0), respectively. Results reported as mean±SEM.

\* Calculated using Student's paired *t* test with results for each substrate normalized by expressing values at 6.9 as a percentage of the corresponding value at 6.0.

was comparable (*P* > 0.05) in incubates from each individual.

**Effect of neutralization of acidic incubates on H<sub>2</sub> production.** Neutralization reversed the inhibition of H<sub>2</sub> production and glucose disappearance demonstrated at acidic conditions and increased the rate of H<sub>2</sub> production from glucose (Fig. 5). At neutral conditions (pH 7.1), 22.2 μmol H<sub>2</sub> were produced and 69.0 μmol

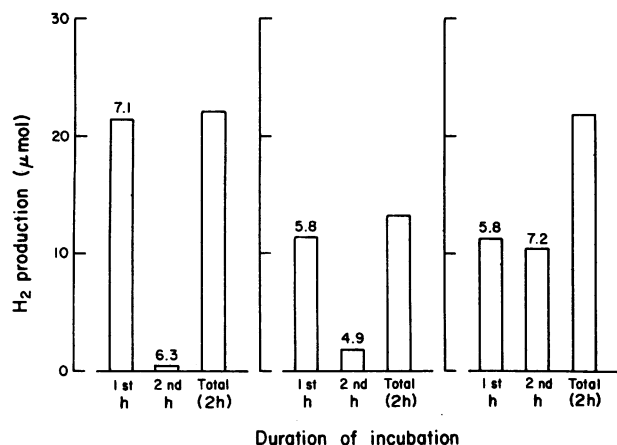


FIGURE 5 Effect of pH on rate of H<sub>2</sub> production and total H<sub>2</sub> produced from glucose by colonic bacteria. A fecal specimen from an adult was divided into three. Left panel indicates specimen initially incubated at neutral pH (control). Middle panel indicates specimen incubated at acidic pH and reincubated at acidic pH. Right panel indicates specimen incubated at acidic pH, neutralized at the end of 1 h, and reincubated. H<sub>2</sub> production per 1 h for each incubate is shown by the bars, and pH at the start of incubation is indicated above the bars. The third bar in each panel represents the total 2-h H<sub>2</sub> production.

glucose disappeared per 2 h. Of these totals, 98% of H<sub>2</sub> production and 98% of glucose disappearance over a 2-h period occurred in hour 1 compared with 52 and 56% for H<sub>2</sub> and glucose, respectively, when a duplicate sample was incubated at pH 5.8. Upon neutralization of the acidic incubate and reincubation for a 2nd h, total H<sub>2</sub> production was 21.8 μmol and glucose disappearance was 65 μmol, thus closely approximating the values obtained under the initially neutral conditions. When expressed as a ratio, 0.32 μmol H<sub>2</sub>/μmol glucose was evolved in the neutral incubation while 0.34 μmol H<sub>2</sub>/μmol glucose was evolved after reincubation of the neutralized incubate. These amounts are comparable to the 0.39 μmol H<sub>2</sub>/μmol glucose evolved in the previously described incubations with fecal homogenates from adults.

**Effect of osmolality on H<sub>2</sub> production in vitro.** H<sub>2</sub> production was pH dependent and independent of osmolality in the range of 200–450 mosmol/kg H<sub>2</sub>O at incubate pH 5.3–6.9 (Fig. 6). H<sub>2</sub> production was, however, affected by conditions below this range of osmolality.

## DISCUSSION

The results indicate that H<sub>2</sub> production from carbohydrate substrates by fecal flora from non-CH<sub>4</sub> producing

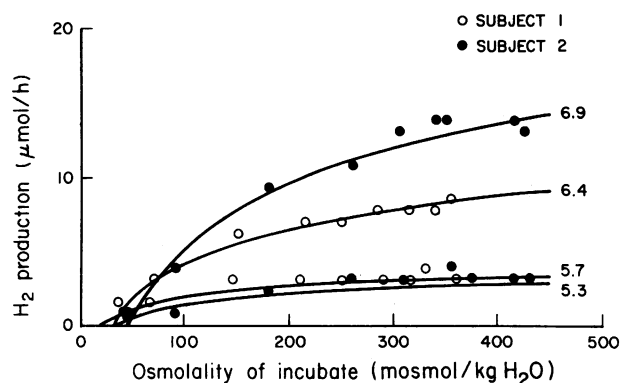


FIGURE 6 Effect of osmolality on H<sub>2</sub> production. Fecal specimens from two adults were homogenized in phosphate-saline solutions and incubated in 55 μmol glucose. Distilled water was added to incubates in amounts ranging from 0.05 to 10.0 ml to achieve a range of osmolality. The initial pH of each incubate is indicated. Curves were drawn by the method of least squares fit. The equations of linear regression for the points between 200 and 450 mosmol/kg H<sub>2</sub>O are:

$$y = 0.011x + 3.91 \text{ (subject 1; pH 6.4)}$$

$$y = 0.003x + 1.70 \text{ (subject 1; pH 5.7)}$$

$$y = 0.001x + 9.03 \text{ (subject 2; pH 6.9)}$$

$$y = 0.001x + 2.49 \text{ (subject 2; pH 5.3)}$$

The 95% confidence limits of the slope include zero except for subject 1; pH 6.4 where the slope (0.011) approaches zero.

individuals is maximal at neutral pH and is strongly inhibited at acid pH. Carbohydrate disappearance and H<sub>2</sub> production are highly correlated at pH varying from 5.5 to 7.6. Inhibition of H<sub>2</sub> production from carbohydrate also occurs at alkaline pH outside the physiologic range. These observations extend previous findings (9) indicating that colonic organisms are metabolically less active at low pH as evidenced by inhibition of ammonia generation.

In vitro incubation systems have been previously validated for the modeling of bacterial metabolism in the rumen (18) and in the human colon (19). Despite inherent limitations such as lack of bicarbonate secretion and absorption of organic acids which occur in vivo, short-term (1 h) incubations as used in the present studies have been shown to reflect accurately the rate of in vivo fermentation reactions (18, 20).

Inhibition of H<sub>2</sub> production does not appear to be due to changes in fecal flora, induction of bacterial H<sub>2</sub> catabolism, or changes in incubate osmolalities. Inhibition of carbohydrate disappearance and H<sub>2</sub> production could not be secondary to quantitative changes in bacterial flora in the in vitro system, since rapid and complete reversal of inhibition could be achieved by neutralization of acidic incubates. Although colonic bacteria have been shown to use as well as to produce H<sub>2</sub> (21), the observation that the total amount of H<sub>2</sub> produced during a 2-h period was comparable in incubates with initially acidic pH which were neutralized after the 1st h of incubation, and in initially neutral duplicate incubates, indicates that the decrease in H<sub>2</sub> measured at acidic pH was not due to increased bacterial utilization of H<sub>2</sub> for metabolic processes.

Inhibition of H<sub>2</sub> production at acidic pH was not mediated by changes in osmolality during the 1-h in vitro incubation period. Changes in osmolality were minimal, and occurred over a range where osmolality had no effect on H<sub>2</sub> production. From these data, it appears that H<sub>2</sub> production from malabsorbed carbohydrate in vivo should likewise not be affected by the osmolality of the colonic environment under conditions of carbohydrate malabsorption. Christopher and Bayless (22) have demonstrated that the osmolality of fecal contents after oral administration of lactose in lactose malabsorbers ranged from ~350–425 mosmol/kg. The present data indicate that, at a given pH, H<sub>2</sub> production by colonic bacteria is independent of osmolality over a broad range from ~200–450 mosmol/kg.

In vivo inhibition of H<sub>2</sub> production from lactulose or lactose at acidic conditions was observed when colonic contents were acidified by addition of lactulose to the diet. The observed decline in H<sub>2</sub> excretion is similar to the reported decrease in H<sub>2</sub> production by rats fed a high legume diet for 4 d (23). Although fecal pH data were not reported, the high content of nonabsorbable sugars present in beans is likely to have reduced colonic pH.

The present data offer considerable indirect evidence that the observed decrease in H<sub>2</sub> excretion at lower colonic pH produced by addition of nonabsorbable carbohydrate to the diet for 1 wk was mediated by a metabolic effect rather than the pH-mediated growth of non-H<sub>2</sub> producing acidophilic organisms. In the present study, H<sub>2</sub> concentration in breath immediately before a lactulose test dose was measurable and not significantly different on day 8 of the lactulose maintenance period compared with day 1, indicating that a H<sub>2</sub>-producing bacterial flora persisted over the 1-wk period. Neutralization of colonic contents by withholding lactulose for 24 h upon concluding the maintenance period caused a rise in H<sub>2</sub> excretion in response to a test dose to the level observed immediately before achievement of acidification in the individual monitored daily. This demonstrates rapid and complete reversibility of inhibition, analogous to the rise in H<sub>2</sub> production observed in vitro by neutralization of acidity in fecal incubates. Quantitative studies of fecal flora were not performed in the present investigations because of potential difficulty in interpretation. For example, significant changes in numbers of specific organisms in feces would not necessarily imply the reduction of H<sub>2</sub>-producing organisms in the whole colon to quantities insufficient to metabolize the lactulose test dose by a H<sub>2</sub>-producing process. Alternatively, it may be speculated that a seemingly insignificant change in number or ratio of organisms could be indicative of a major alteration in H<sub>2</sub>-yielding reactions in the colon.

The primacy of the pH effect on H<sub>2</sub> production in vivo is further supported by the temporal association between achievement of fecal acidification and inhibition of H<sub>2</sub> production demonstrated in the individual monitored daily during the lactulose maintenance period, and in the immediate inhibition of the H<sub>2</sub> response to lactose when acidification was achieved rapidly by administration of higher dose lactulose for 24 h. Moreover, inhibition of the breath H<sub>2</sub> response associated with intake of lactulose did not appear attributable to its laxative effects. Laxation induced by magnesium sulfate, which in contrast to lactulose does not acidify the colon, did not inhibit H<sub>2</sub> excretion following a lactulose test dose. In fact, a twofold increase in the rate of H<sub>2</sub> excretion compared with the control study was seen; this could possibly be attributed to the 0.8 U rise in fecal pH accompanying magnesium sulfate administration, which would be consistent with the finding of significant alkalization of the distal colon by magnesium sulfate previously demonstrated by Bown et al. (15). This explanation assumes that a portion of the H<sub>2</sub> excreted is produced in the distal colon. It is noteworthy that the present in vitro studies (Fig. 4) would predict a twofold increase in H<sub>2</sub> production between the observed pre- and postmagnesium sulfate fecal pH values.

Inhibition of the breath H<sub>2</sub> response associated with

the administration of non- or malabsorbed carbohydrate has previously been observed in children with acute enteritis, and in clinical monosaccharide intolerance associated with intractable diarrhea of infancy (24). No stool pH measurements were reported, but these conditions generally produce acidic fecal contents (12, 25). It is likely that the reported inhibition of H<sub>2</sub> production associated with large quantities of unmetabolized carbohydrate in stool (24) was due at least in part to the effects of pH described in the present study, in addition to potential changes in total colonic flora from diarrhea and to decreased transit time.

The present data raise several issues regarding the clinical use and interpretation of breath H<sub>2</sub> tests for detection of carbohydrate malabsorption. Since these experiments indicate that the presence of acidic colonic contents influences breath H<sub>2</sub> excretion after test doses of lactulose or lactose, maximal sensitivity requires avoidance of breath H<sub>2</sub> tests until fermentable substrate has been eliminated from the diet, or until the patient has sufficiently recovered from acute enteritis, long enough to allow the fecal pH to rise. Whereas the *in vitro* studies indicate that the pH optimum for H<sub>2</sub> production occurs at neutral pH and that H<sub>2</sub> production is virtually eliminated at pH approaching 5.0, a specific pH above which a rise in H<sub>2</sub> excretion occurs in response to malabsorbed carbohydrate cannot be established with certainty from these data. Moreover, attempts to identify a minimum stool pH above which H<sub>2</sub> breath tests can be interpreted by current criteria must also take into account that fecal pH may not accurately reflect the pH more proximally in the colon (15).

In addition to differences in fecal microflora and pulmonary factors, variations among individuals in colonic pH are likely to contribute to individual differences in the breath H<sub>2</sub> response to identical amounts of the nonabsorbable substrate lactulose observed by us and others (16). The large differences between populations in the percentage of those failing to manifest a rise in breath H<sub>2</sub> despite administration of lactulose, e.g., 20% in Israel (26) vs. 2% in the U. S. (27), may be due to differences in fecal pH caused by regional diets.

These individual differences in H<sub>2</sub> responses have precluded the use of absolute H<sub>2</sub> levels after administration of malabsorbed substrate as a quantitative measure of carbohydrate malabsorption. However, Bond and Levitt (16) have demonstrated that H<sub>2</sub> excretion after administration of a standard dose of lactulose to a subject can be used to calculate the volume of H<sub>2</sub> expected when an identical amount of another test sugar is not absorbed by the same subject. Thus, the exact amount of a nutrient carbohydrate such as lactose escaping absorption can be derived by determination of H<sub>2</sub> produced after sequential administration of equivalent amounts of nonabsorbable and potentially absorbable carbohydrate. The present study indicates that

equivalent amounts of H<sub>2</sub> are produced from different carbohydrate substrates by fecal flora from individual neonates. Accordingly, this methodology may be applicable for the quantification of carbohydrate absorption in newborns, an age group from which reliable information regarding carbohydrate absorption is meager. The present studies indicate that the accuracy of this methodology would be greatest when the pH of the colon at the time the test substrate is given equals the colonic pH at the time the lactulose is given. The use of hydrogen measurements in this manner to quantify carbohydrate absorption also requires that the colonic pH not be so low as to inhibit H<sub>2</sub> production totally. Further studies are needed to validate this approach.

Methane-excreting individuals were not included in these studies because CH<sub>4</sub> appears to be produced from H<sub>2</sub> by colonic organisms (13), raising the possibility that the H<sub>2</sub> response to nonabsorbable carbohydrate may be altered in this population. The observations reported here suggest that the effects of pH on H<sub>2</sub> excretion in methane excretors, representing approximately one-third of the U.S. population (28), should be further investigated.

The colon has been shown to play an important role in reducing the osmotic load and conserving the energy value of malabsorbed carbohydrate by absorption of fermentation products derived from intracolonic bacterial metabolism of sugars (29, 30). The present data suggest that this process is pH dependent. Thus, the observed inhibition of bacterial fermentation at acidic pH and the consequent persistence of large amounts of glucose in the incubate are consistent with the possibility that pH is an important factor in the efficiency with which colonic bacteria help conserve malabsorbed carbohydrate.

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