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Acetate Production from Hydrogen and [^{13}C]Carbon Dioxide by the Microflora of Human Feces

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Fecal suspensions from humans were incubated with $^{13}CO_2$ and H_2. The suspensions were from subjects who harbored 10^8 and 10^{10} methanogens per g (dry weight) of feces, respectively, and from a subject who did not harbor methanogens. Quantitative nuclear magnetic resonance spectroscopy showed that acetate labeled in both the methyl and carboxyl groups was formed by suspensions from the subject without methanogens and the subject with the lower concentrations of methanogens. The amounts of labeled acetate formed were in agreement with the amounts expected based on measurements of H_2 utilization. No labeled acetate was formed by suspensions from the subject with the higher concentrations of methanogens, and essentially all of the H_2 used was accounted for by CH_4 production. Suspensions from the subject with lower concentrations of methanogens produced both methane and acetate from H_2 and CO_2. The results indicate that reduction of CO_2 to acetate may be a major pathway for microbial production of acetate in the human colon except when very high concentrations of methanogens (ca. 10^{10} per g [dry weight] of feces) are present. Double-labeled acetate was also formed from H_2 and $^{13}CO_2$ by fecal suspensions from nonmethanogenic and moderately methanogenic rats.

Plant cell wall carbohydrates (cellulose, hemicellulose, and pectin) are important constituents of food, particularly when diets are high in fiber. They are not digested by enzymes synthesized by humans. Complex eubacterial microbial communities in the colon of humans and other monogastric animals use these carbohydrates as major substrates for a fermentation that produces mainly acetic, propionic, and butyric acids and H_2 and CO_2 (5, 20).

Methanogens are often present in the colon of monogastric animals (15). These archaebacteria use the H_2 formed by the eubacterial community to produce CH_4. Two species have been found in the human colon (15). The methanogen responsible for the production of significant amounts of colonic CH_4 is *Methanobrevibacter smithii*, which uses H_2 to reduce CO_2 to CH_4 (12, 18). A less numerous species, *Methanosphaera stadtmaniae*, uses H_2 to reduce methanol to CH_4 and is incapable of producing CH_4 from CO_2 (14). Some people have essentially no CH_4 formed in their colon, whereas others have constant daily production of several liters a day (1) because the concentration of *Methanobrevibacter smithii* varies from <10 to 10^{10} per g (dry weight) of feces (18). Individuals with 10^8 to 10^{10} *M. smithii* per g (dry weight) produce approximately 0.03 to 3 liters of methane per day and have detectable (>1 ppm [>1 μl/liter]) breath CH_4 (15). About 80% of the CH_4 and net H_2 produced in the colon is eliminated as flatus, and about 20% is transported to the blood and eventually eliminated in expired breath (1). For every liter of CH_4 produced daily, there are 4 liters of H_2 produced and used in the colon. However, there is net daily production of about 1 liter of H_2 in the colon whether or not CH_4 is a significant product (1). Either much less H_2 is produced in the absence of significant methanogenesis or there is an alternative use of the electrons that are used for the reduction of CO_2 to CH_4. Since the use of H_2 to reduce CO_2 to acetate has been shown to occur in the ceca of rats (16), Wolin and Miller hypothesized that this alternative path of CO_2 reduction was a major human colonic process in the absence of significant methanogenesis (20). Therefore, we measured the production of acetate from CO_2 and H_2 by the microflora of human feces and the incorporation of $^{13}CO_2$ into acetate. The results presented in this report indicate that the production of acetate from H_2 and CO_2 is an active process in humans who are not highly "methanogenic." We also present results of similar experiments conducted with the fecal flora of methanogenic and nonmethanogenic strains of rats.

MATERIALS AND METHODS

Human subjects. Human subjects were in their mid-20s to mid-30s and volunteered to participate in the study. All subjects consumed normal Western diets, and none had any diagnosed colonic disease or other health complaints. Subjects B and D participated in earlier studies of human fecal methanogenesis (12, 13). Human fecal fermentation studies were initially approved by the New York State Department of Health Institutional Review Board in January 1982, and protocols were reviewed and approved yearly thereafter.

Animals. Wistar and DA strain rats were obtained from colonies maintained by the central animal facility of the Wadsworth Center. The Wistar colony was established in 1959 with animals obtained from the Walter Reed Army Hospital and have been maintained by random breeding. The DA rats were originally obtained from R. N. Smith, Case Western Reserve University, Cleveland, Ohio, and the colony has been maintained at the Wadsworth Center by random breeding. The animals used in this study were 9- to 18-month-old males. All animals were fed commercial laboratory rat chow and water ad libitum and maintained on a 12-h light-dark schedule. The animals were transferred to raised wire cages for ca. 1 h for collection of fresh fecal

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pellets. Rat fecal fermentation studies were initially approved by the Wadsworth Animal Welfare Committee in June 1983, and protocols were reviewed and approved yearly thereafter.

Enumeration procedures. The anaerobic techniques and media for enumeration of total viable anaerobe and methanogen concentrations in feces were as described by Miller and Wolin (11, 12). The 10^{-1} dilution was prepared by the stomacher method (13). Serial dilutions (10-fold) were prepared in anaerobic dilution solution (3). For enumerations with human feces, all media and dilution solution were reduced with 1.25% (each) cysteine hydrochloride and $Na_2S \cdot 10H_2O$. For enumerations with rat feces, media and dilution solution were reduced with 1.25% (each) dithiothreitol and $Na_2S \cdot 10H_2O$. For total anaerobe counts, 0.5 ml of each appropriate dilution was inoculated in duplicate roll tubes containing a complex medium with 10% rumen fluid and agar and a 100% CO_2 atmosphere. Methanogens were enumerated in a similar fashion in the complex medium with the addition of cephalothin and clindamycin and an 80% H_2–20% CO_2 atmosphere. All incubations were at 37°C. At 14 days, roll tube colonies were counted and portions of the headspace of antibiotic-containing roll tubes were analyzed for methane. The remainder of the 10^{-1} dilution (ca. 10 ml) was used for enrichment of methanogens. The bottles were regassed and pressurized to 203 kPa with 80% H_2–20% CO_2, incubated for 14 days, and analyzed for methane. Dry fecal weights were determined as described previously (12).

Production of acetate from $^{13}CO_2$. Suspensions, 10% (wt/vol), of freshly collected feces were prepared in anaerobic dilution solution in which $NaH^{13}CO_3$ (99% enriched) were substituted for $NaHCO_3$ (natural abundance of ^{13}C, 1.1%). Suspensions were mixed in a stomacher (Lab-Blender 80; Tekmar Co., Cincinnati, Ohio) for 30 s. After 5 ml was dispensed into duplicate serum bottles (ca. 100 ml) of known calibrated volume under N_2, the atmosphere of one bottle was replaced with H_2, a butyl rubber stopper was inserted, and the bottle was then sealed with an aluminum seal. To avoid overpressure, a syringe was inserted to collect and discard displaced gas. The initial H_2 concentration was calculated from the difference of the liquid volume and the calibrated volume of the closed serum bottle. The second bottle was sealed under N_2 in a similar fashion. The remainder of the fecal suspension was centrifuged, and the supernatant was used to determine the concentrations of endogenous soluble compounds.

Addition of $^{13}CO_2$ to make the atmosphere 20% $^{13}CO_2$ was accomplished by adding H_2SO_4 to a solution containing the stoichiometric amount of labeled $NaHCO_3$ necessary for generation of the volumetric requirement for $^{13}CO_2$. We used three plastic syringes connected with a three-way stopcock (Popper & Sons, Inc., New Hyde Park, N.Y.) to generate the CO_2. Bicarbonate solution was syringed into a 50-ml syringe from a 5- or 10-ml syringe. The stopcock was closed to the solution source and opened between the 50-ml syringe and a 50-ml syringe containing excess acid. Generated gas displaced the rubber-gasketed plunger of the syringe containing bicarbonate. The contents of the two syringes were then mixed by drawing the plunger of the acid syringe back and forth with final collection of all gas in the bicarbonate syringe and all liquid in the acid syringe. The stopcock was closed, the acid syringe with liquid was removed, and the collected gas in the other syringe was added through a 0.2-μm filter to the serum bottle containing the fecal suspension. The filter removed any traces of liquid that remained in the syringe containing the labeled CO_2. All additions were made while the suspensions were held on ice. Control fermentations with an N_2–$^{13}CO_2$ gas phase were prepared in an identical fashion.

After addition of labeled CO_2, suspensions were rapidly warmed to 37°C, incubated for 24 h with shaking (50 rpm), and terminated by the addition of 5 N H_2SO_4 (0.2 ml). The suspensions were then equilibrated to room temperature, excess gas pressure was measured by displacement of the plunger of an inserted syringe, and portions of the headspace gas were removed for quantitation of H_2 and CH_4 as described below. After quantitation of gases, the bottles were gassed with N_2 for 10 min. Acidified suspensions were centrifuged at 12,062 × *g* for 10 min, and the supernatants were analyzed for fermentation products by high-pressure liquid chromatography.

NMR methods. Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker WH 90 FT spectrometer and a Varian XL 300 spectrophotometer operating at 22.64 and 75.43 MHz, respectively. Pulses of 60 s were used, and the recycling time was 2 s. For the WH 90 experiments, broadband ^1H decoupling was used, and for the XL 300 experiments, Waltz decoupling was used. The number of transients acquired ranged from 1,200 to 33,000, although usually 1,200 were sufficient. The NMR locking material was deuterium oxide, and the internal standards were dimethyl sulfoxide (DMSO) and dioxane. DMSO was purified by heating with calcium hydride and distillation.

Quantitative calibration plots were developed by using singly labeled ^{13}C sodium acetate enriched with ^{13}C at either C-1 or C-2 in appropriate combinations with unlabeled sodium acetate. Eighteen standard solutions were prepared in D_2O. Nine solutions were labeled in the C-1 position and nine were labeled in the C-2 position. The concentrations of ^{13}C label ranged from 1.1 (natural abundance) to 99% enriched. A calibrated quantity of internal standard (DMSO, dioxane, or both in some instances) was added to each standard solution. The standard solution was added to a 10-mm NMR tube, and 1,200 transients were acquired under identical spectral conditions. The DMSO peak at 40.6 ppm or the dioxane peak at 67.3 ppm or both were used as references. The singly labeled carbonyl had a chemical shift of 177.4 ppm and the singly labeled methyl had a peak at 21.2 ppm, which agreed well with literature values (17). The intensities of the labeled acetate peaks and the internal standards were determined by integration. Plots of the ratio of peak intensities of C-1 or C-2 to internal standard versus label concentration gave straight lines. For a given concentration of labeled ^{13}C, the peak intensity ratio of the methyl carbon to standard was 1.73 times greater than that of the carbonyl due to NOE and relaxation time differences. Doubly labeled sodium acetate gave an AX pattern (^2J = 60 Hz) with doublets at each position and chemical shifts and peak intensity ratios in excellent agreement with those of the singly labeled materials.

NMR analysis of fermentation samples. Acidified fermentation supernatant (1 ml) was diluted with 1 ml of D_2O, and calibrated quantities of DMSO and dioxane were added. The concentrations of singly and doubly labeled acetates were determined by integration of the relevant labeled peaks and the internal standards and by the calibration plots described above. Formate was identified by comparison of chemical shifts with literature values (17).

Other analytical methods. Soluble fermentation products were determined by high-pressure liquid chromatographic procedures (6). We used an Aminex ion-exclusion column (HPX-87H; Bio-Rad Laboratories, Richmond, Calif.) at 35°C

TABLE 1. Total viable anaerobes and methanogen concentrations in human and rat feces

Fecal source	% Dry fecal wt	Log_{10} per g (dry wt) of feces	
		Total anaerobes	Methanogens
Humans			
B	22.9	11.3	10.1
D	23.7	11.6	7.7
J	28.7	11.4	<2
Rats			
Wistar	51.4	10.7	9.1
DA	35.6	10.4	<2

and eluted with 0.013 N H_2SO_4 (0.55 ml/min; 77 kg of F per cm^2). Compounds were detected by refractive index, identified and quantified with a Shimadzu C-R3A integrator (Shimadzu Scientific, Baltimore, Md.) and by the absolute calibration curve method. H_2 and CH_4 were quantified by described gas chromatographic procedures (4).

Materials. ^{13}C-labeled C-1 and C-2 and doubly labeled sodium acetate were from MSD Isotopes, Montreal, Quebec, Canada, and were 99% enriched. ^{13}C-labeled sodium bicarbonate (99% enriched) was purchased from Cambridge Isotope Laboratories, Woburn, Mass. All other chemicals were of reagent grade or better.

RESULTS

Enumeration of feces. The dry matter, methanogen, and total viable anaerobe concentrations of feces of the three human subjects are shown in Table 1. The concentrations of viable anaerobes were similar in the three subjects. Subject B had high concentrations of methanogens (10^{10} per g, dry weight), whereas subject D had only moderate concentrations of methanogens (ca. 10^8 per g, dry weight). We showed that the concentrations of viable anaerobes and methanogens in feces of subjects B and D are constant over long time periods (13). Subject J did not have a significant methanogen population. Methane was not detected in the headspace of roll tubes of the lowest plated dilution (10^{-3}) or in 14-day enrichment cultures.

The dry matter, methanogen, and total viable anaerobe concentrations of Wistar and DA rat feces are also shown in Table 1. The two rat strains had similar total viable anaerobe concentrations. However, the total viable anaerobe concentrations were lower than that measured in human feces. The Wistar rat feces contained moderately high concentrations of methanogens, whereas DA rat feces did not contain significant concentrations of methanogens.

Stoichiometry of acetate and CH_4 production from H_2-$^{13}CO_2$. The conversion of H_2 and $^{13}CO_2$ to CH_4 or acetate was investigated with fecal suspensions from the three human subjects. Table 2 shows the amount of H_2 used and the amounts of acetate and CH_4 produced after incubation of the fecal suspensions with H_2 and CO_2. The amounts of acetate and CH_4 formed from endogenous substrates are also shown. Most of the H_2 was used for CH_4 production by suspensions from subject B (Table 2). The stoichiometry of H_2 disappearance and the net acetate formed over that observed in the absence of H_2 suggested that some acetate was produced by CO_2 reduction (Table 3). Small amounts of CH_4 were formed from added H_2 by suspensions from subject D (Table 2). Formate also accumulated. However, formate and CH_4 did not account for all of the H_2 that disappeared (Table 3). The net acetate formed over that observed in the absence of added H_2 suggested that most of the H_2 that disappeared was used for acetate production. All of the H_2 used by the nonmethanogenic suspensions from subject J could be accounted for by the amount of acetate produced (Tables 2 and 3).

TABLE 2. Stoichiometry of H_2-$^{13}CO_2$ fermentation by human and rat feces

Fecal source	Gas phase	Total μmol[a] of:					
		AcH	ProH	BuH	For	H_2	CH_4
Humans							
B	H_2-CO_2	65	13	10	0	−3,237	823
	N_2-CO_2	28	10	7	0	9	41
	Net	37	3	3	0	−3,246	782
D	H_2-CO_2	99	22	12	71	−447	24
	N_2-CO_2	46	17	17	0	0	0
	Net	53	5	−5	71	−447	24
J	H_2-CO_2	139	49	36	0	−187	0
	N_2-CO_2	95	32	17	0	23	0
	Net	44	17	19	0	−210	0
Rats							
Wistar	H_2-CO_2	318	34	34	0	−1,630	235
	N_2-CO_2	116	34	29	0	9	20
	Net	202	0	5	0	−1,639	215
DA	H_2-CO_2	417	50	28	0	−1,052	0
	N_2-CO_2	171	58	25	0	28	0
	Net	246	−8	3	0	−1,080	0

[a] Total per 5 ml of a 10% (wt/vol) fecal suspension incubated for 24 h. AcH, ProH, and BuH are corrected for concentrations present in the initial fecal suspension. AcH, Acetate; ProH, propionate; BuH, butyrate; For, formate. Other organic acids or alcohols were not detected in either initial fecal suspensions or fecal fermentation supernatants.

Results of similar experiments with Wistar and DA rat fecal suspensions are also shown in Table 2. All of the H_2 was apparently used for acetate formation by fecal suspensions from the nonmethanogenic DA strain, whereas approximately equivalent amounts of H_2 were used for methane and acetate formation by the methanogenic Wistar fecal suspensions (Table 3).

Formation of $^{13}CH_3{}^{13}COOH$ from $^{13}CO_2$. Supernatants from the experiments described above were analyzed by high-resolution NMR. Table 3 shows that the reduction of $^{13}CO_2$ with H_2 led to the formation of double-labeled acetate with suspensions from the moderately methanogenic subject D, the nonmethanogenic subject J, the nonmethanogenic DA rat strain, and the methanogenic Wistar rat strain. No ^{13}C-labeled acetate was formed by the fecal suspensions from the highly methanogenic subject B. The total amounts of $^{13}CH_3$, $^{13}COOH$, and double-labeled acetate quantified by NMR were in reasonable agreement with the expected amounts of acetate formed when calculated from residual H_2 that was not used for CH_4 or formate formation (Table 3). With the exception of [^{13}C]formate formed by the suspensions of subject D, no other labeled compounds were detected in the fecal supernatants.

TABLE 3. Formation of [^{13}C]acetate from $^{13}CO_2$ and H_2 by human and rat fecal suspensions[a]

Fecal source	Total μmol							
	H_2 used[b]			Residual H_2	Expected [^{13}C]acetate[c] from:		Observed [^{13}C]acetate	
	Total	For formate production	For CH_4 production		Residual H_2	Net acetate value	Single + double	Double
Humans								
B	3,252	0	3,128	124	31	36	0	0
D	447	71	96	280	70	51	71	45
J	248	0	0	248	62	36	56	37
Rats								
Wistar	1,649	0	860	789	197	202	188	108
DA	1,086	0	0	1,086	272	246	225	147

[a] All calculations are based on net values in Table 2 and are for 5 ml of a 10% (wt/vol) fecal suspension incubated for 24 h.

[b] Total H_2 used was calculated from net H_2 used plus an estimated amount of H_2 produced by the conversion of hexose→butyrate + $2H_2$ + $2CO_2$. Endogenous substrate was assumed to be a hexose. The amount of H_2 used for formate and CH_4 formation was calculated according to (i) $CO_2 + H_2 \rightarrow HCO_2H$ and (ii) $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$.

[c] Expected [^{13}C]acetate was calculated two ways: (i) based on the residual H_2 and $2CO_2 + 4H_2 \rightarrow$ acetate + $2H_2O$; and (ii) based on the observed net acetate minus an estimated amount of acetate produced by the conversion of 1.5 hexose→2 propionate + acetate + CO_2. Endogenous substrate was assumed to be a hexose.

DISCUSSION

Evidence for the colonic production of acetate via a CO_2 reduction pathway includes the finding of stoichiometric conversion of H_2 and CO_2 to acetate by the human fecal suspensions and the determination that acetate formed after incubation with H_2 and $^{13}CO_2$ is labeled as expected if CO_2 is the precursor of both carbons of acetate. Reduction of CO_2 to acetate with H_2 as the electron donor was shown previously with rat cecal contents by Prins and Lankhorst (16) and with termite gut contents by Breznak and Switzer (2). However, it is necessary to demonstrate that significant incorporation of CO_2 into acetate occurs during the fermentation of colonic substrates such as cellulose and hemicelluloses to verify that the pathway is an important source of acetate in colonic fermentations. If so, it will be necessary to evaluate the significance of H_2 as an electron donor for CO_2 reduction to acetate. Whereas colonic CH_4 formation depends on the production of H_2 by fermentation by nonmethanogenic microorganisms followed by reduction of CO_2 by methanogens, CO_2 reduction to acetate may not necessarily involve analogous interactions. It is well known that several species of bacteria produce a homoacetate fermentation of carbohydrates; e.g., 1 mol of glucose is fermented to 3 mol of acetate (9). Reduction of CO_2 to acetate with H_2 as an electron donor may be an activity of similar microorganisms in the colonic ecosystems that is not actually expressed during the fermentation of colonic substrates; i.e., H_2 may not be a true intermediate in the formation of acetate from CO_2. Alternatively, the production of some of the acetate formed from colonic substrates could involve the production of H_2 by fermentation by some microbial species and the use of H_2 by different species that reduce CO_2 to acetate.

Although some acetate was formed when suspensions of the highly methanogenic subject B were incubated with H_2 and CO_2, no $^{13}CO_2$ was incorporated into acetate. We have no explanation for the source of the unlabeled acetate. It is clear that CH_4 is the major product of CO_2 reduction in subject B. The reduction of $^{13}CO_2$ to [^{13}C]acetate by subjects D and J was approximately 8% of the amount of CO_2 reduction to CH_4 by subject B. This is probably a minimal value because the data used for the calculations were from endpoint rather than rate studies. If corrections are made for dry weight differences, the reduction of CO_2 to acetate was about 1.5 and 3 times higher for Wistar and DA rats, respectively, than for human subjects D and J. Again, these are probably minimal values.

The accumulation of [^{13}C]formate in the subject D incubations appears to be due to direct reduction of CO_2 by H_2. No formate accumulated in the absence of H_2. Formate is the intermediate precursor of the methyl group of acetate when acetate is formed from CO_2 (9). Presumably, the flora of subject D reduced CO_2 to formate more rapidly than it was transformed to acetate and more rapidly than CO_2 was used by the methanogenic flora for production of methane.

Pure cultures of *Peptostreptococcus productus* and *Eubacterium limosum*, known human colonic carbohydrate-fermenting species, have been shown to use H_2 and CO_2 as growth substrates (7, 8, 10). They or unknown CO_2-reducing acetogens may interact to increase the production of acetate by H_2-forming, carbohydrate-fermenting species, as has been shown for other fermentative organisms grown with H_2-using species (19). High acetate concentrations in the human large bowel may be due to interspecies H_2 transfer between fermentative species and acetogenic species that use H_2 to reduce CO_2 to acetate or methanogens that use H_2 to reduce CO_2 to CH_4. However, since the reduction of CO_2 to acetate can be either an interspecies or an intraspecies process, additional studies are necessary to evaluate the significance of the reduction in the formation of acetate from the major substrates of the colonic fermentation.

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