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Pathways of Acetate, Propionate, and Butyrate Formation by the Human Fecal Microbial Flora

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The pathways of short-chain fatty acid (SCFA; acetate, propionate, and butyrate) formation from glucose were determined for the human fecal microbial communities of two subjects. The pathways were identified by radioisotope analysis of the SCFA and CO_2 obtained after incubation of fecal suspensions with glucose under 20% CO_2 with [1-^{14}C]glucose, [3,4-^{14}C]glucose, or $^{14}CO_2$. Acetate was chemically degraded to learn the labeling of the methyl and carboxyl carbons. The labeling of CO_2 and acetate showed that the major route of glucose catabolism was the Embden-Meyerhof-Parnas pathway, with production of CO_2 from pyruvate carboxyl carbon. Labeling of the methyl and carboxyl carbons of acetate by $^{14}CO_2$ or [3,4-^{14}C]glucose proved that acetate was formed from CO_2 by the Wood-Ljungdahl pathway. CO_2 reduction accounted for about one-third of the acetate formed by suspensions from subject 1 and about one-fourth of the acetate formed by suspensions from subject 2. Propionate was formed by a CO_2 fixation pathway, and butyrate was formed by classical routes of acetyl-*S* coenzyme A condensation. The amount of CO_2 formed from [1-^{14}C] glucose and acetate labeling patterns obtained with the other ^{14}C precursors indicated that the Entner-Doudoroff, transketolase-transaldolase, and heterolactic pathways were not significant. Fermentation of cabbage cellulose by subject 1 followed the same pathways as were used for glucose. The results with suspensions from subject 2 suggested that some radioactive acetate was formed from the C-3 of glucose by the *Bifidobacterium* pathway.

The short-chain fatty acids (SCFA) acetate, propionate, and butyrate are major products of the microbial fermentation of plant fiber polysaccharides in the human colon (2, 23). The SCFA have an impact on the metabolism of the host (13). Acetate and propionate influence cholesterol production (21), and propionate is gluconeogenic (21). Butyrate is a major source of energy for colonic epithelial cells (12), and low concentrations cause differentiation of mammalian cells as well as colon carcinoma cells (6, 16).

The pathways of formation of the SCFA from carbohydrates have not been determined for the human colonic ecosystem. Information about the pathways used by the colonic microbial community can provide information about the metabolism of the predominant species and variations in the community and its metabolism that might relate to diet or disease. This report presents the results of investigations of the pathway of glucose fermentation by fecal suspensions from two healthy humans. The pathways were elucidated by radioisotope analysis of the SCFA obtained after incubations of the suspensions with glucose under 20% CO_2 with [1-^{14}C]glucose, [3,4-^{14}C]glucose, or $^{14}CO_2$.

MATERIALS AND METHODS

Fecal suspensions. Fecal samples were obtained from two healthy adults with no history of diagnosed large bowel disease. Subject 1 participated in previous studies of fecal microbial community fermentation (19, 20). Human fecal fermentation protocols were reviewed and approved by the New York State Department of Health Institutional Review Board.

A 10% (wt/vol) suspension of feces was prepared in anaerobic dilution solution as described previously (19). The suspensions were held at 4°C and used within 24 h of collection. Anaerobic conditions were maintained by use of the serum bottle modification of the Hungate technique (8).

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Fermentations. All radioactive compounds were obtained from New England Nuclear, Boston, Mass. [1-^{14}C]glucose, [3,4-^{14}C]glucose, and $NaH^{14}CO_3$ solutions were prepared in boiled and cooled distilled water, filter sterilized (0.2-μm-pore-size filter), and stored under N_2 at −20°C. [1-^{14}C]glucose (1 μCi), [3,4-^{14}C]glucose (1 μCi), and $NaH^{14}CO_3$ (2 μCi) were added to separate tubes containing 5 ml of fecal suspension amended with 278 μmol of nonradioactive glucose. A fecal suspension from subject 1 was also incubated with 50 mg of cellulose purified from cabbage (20) and $NaH^{14}CO_3$ (2 μCi). The gas phase was 80% N_2–20% CO_2 (101 kPa). Fermentation tubes were incubated with rotation at 37°C for 24 h. Fermentation suspensions were acidified with 50 μl of 5 N H_2SO_4 per ml of suspension. The total volume of gas produced during the fermentation was determined by adding the volume of gas displaced into an inserted plastic hypodermic syringe with a sealed rubber plunger tip to the predetermined gas headspace volume of the fermentation tube. After analysis for H_2, CH_4, and CO_2, portions of the gas phase were removed for radioactivity measurements. The fermentation suspension was transferred to a fume hood, and the suspension was sparged with CO_2 for 10 min. The suspension was centrifuged at 1,397 × *g* for 10 min. The supernatant was passed through a 0.22-μm-pore-size filter and stored at −20°C until further analysis.

Analytical methods. Glucose and fermentation products were determined by high-pressure liquid chromatographic (HPLC) procedures described by Ehrlich et al. (3). The injection volume was 20 μl, and the column was an Aminex HPX-87H (Bio-Rad Laboratories, Rockville Center, N.Y.) heated to 35°C. The eluent was 0.013 N H_2SO_4, and the flow rate was 0.55 ml/min at a pressure of 60 kg/cm^2. Eluting compounds were detected by refractive index and identified and quantified with a Shimadzu Chromatopac C-R3A integrator data processor (Shimadzu, Columbia, Md.) and by the absolute calibration curve method. Hydrogen, CH_4, and CO_2 were measured by gas chromatography as described by Pavlostathis et al. (11).

Radioactivity of CO_2 and acid products. The amount of radioactivity in CO_2 was determined by absorption into phenethylamine (Eastman Kodak, Rochester, N.Y.) as described by Miller and Wolin (9). Acetate, propionate, and butyrate were purified from 100 μl of acidified fermentation supernatant by the HPLC procedure described above. Portions were removed to measure the amount of supernatant radioactivity recovered in the acetate, propionate, and butyrate fractions. The recovery of radioactivity in the acids obtained from the HPLC columns varied from 79 to 105% of the amount of activity applied to the columns. The radioactivity of the acids was corrected for the efficiency of recovery, and the corrected radioactivity was used for calculations.

The radioactivity of the carboxyl and methyl carbons of the purified acetate was determined by sequential oxidation to CO_2 by the Schmidt procedure as described previously (4, 9). Schmidt degradation of authentic [U-^{14}C]acetate yielded equivalent radioactivity from each of the carbon atoms and a combined recovery of 96% of the initial radioactivity.

Radioactivity measurements. The amounts of radioactivity of stock solutions, acidified fermentation supernatants, purified acetate, propionate, butyrate, and

TABLE 1. Amounts of products from glucose[a]

Product	Subject 1		Subject 2	
	Total μmol formed	% of total SCFA	Total μmol formed	% of total SCFA
Acetate	398	70	274	51
Propionate	111	20	148	28
Butyrate	58	10	117	22
Hydrogen	0	0	109	0

[a] The glucose added (278 μmol) was completely used. Methane was not produced.

CO_2 absorbed into phenethylamine were determined in Aquasol (New England Nuclear, Boston, Mass.) by using a scintillation counter (model 1217 Rackbeta; LKB Wallac, Turku, Finland). Radioactivity values were corrected for quenching by the external standard method.

RESULTS AND DISCUSSION

Glucose fermentation products. The amounts of SCFA formed after incubation of the fecal suspensions with glucose are shown in Table 1. The results are the means of the values obtained from the three incubation mixtures with the different labeled precursors. The suspensions fermented all of the glucose during the incubation period. The suspension from subject 2 produced lower proportions of acetate and higher proportions of propionate and butyrate from glucose. No other products were detected by HPLC analysis, which would have detected ca. 1 mM ethanol, lactate, and succinate and other common carbohydrate fermentation products. Valerate and branched-chain volatile fatty acids, products of amino acid catabolism, are usually only 5% of the total short-chain volatile acids in feces (18) and were not determined in these experiments. Neither subject's suspensions produced CH_4. Hydrogen accumulated as a product of glucose fermentation by the suspension from subject 2.

Radioactivity of CO_2. Table 2 shows the specific radioactivity of CO_2 obtained after incubation with the different radioactive precursors. For both subjects, the amount of CO_2 formed from the C-3 and C-4 positions of glucose was 9 to 10 times greater than the amount formed from the C-1 position. This indicates fermentation of glucose by the Embden-Meyerhof-Parnas (EMP) pathway and production of CO_2 from the carboxyl groups of pyruvate formed from C-3 and C-4 of glucose. The amount of CO_2 formed from [1-^{14}C]glucose (ca. 6% of the radioactivity) might represent a small amount of catabolism via pathways that require decarboxylation of 6-phosphogluconate or anabolic decarboxylation by pathways that generate pentose needed for biosynthetic reactions. The patterns of labeling of the acetate obtained with the different ^{14}C precursors (see below) also indicate only minor contributions of the Entner-Doudoroff, transketolase-transaldolase, and heterolactic pathways to the fermentation of glucose by the suspensions.

Radioactivity of acetate. Table 2 shows the specific radioactivity of acetate obtained after incubation with the different radioactive precursors. Acetate production from C-1 was 2.8 and 3.8 times greater than the amount from the C-3 and C-4 positions of glucose for subjects 1 and 2, respectively. Significant amounts of acetate were formed from CO_2 by suspensions from both subjects. The acetate formed was isolated by HPLC and subjected to Schmidt degradation. Table 3 shows that the methyl and carboxyl positions of acetate were almost equally labeled when the substrate was $^{14}CO_2$ or [3,4-^{14}C]glucose. This shows that both carbon atoms of acetate are formed from CO_2 by the Wood-Ljungdahl pathway (23).

TABLE 2. Radioactivity of CO_2 and acetate

^{14}C precursor	Radioactivity (dpm/μmol)			
	Subject 1		Subject 2	
	CO_2	Acetate	CO_2	Acetate
[1-^{14}C]glucose	163	2,963	152	2,917
[3,4-^{14}C]glucose	1,433	1,044	1,497	764
$^{14}CO_2$	3,411	2,132	3,527	1,113

TABLE 3. Radioactivity of methyl and carboxyl carbons of acetate

^{14}C precursor	Radioactivity (dpm/μmol)					
	Subject 1			Subject 2		
	CH_3	COOH	Ratio, CH3/COOH	CH_3	COOH	Ratio, CH_3/COOH
[1-^{14}C]glucose	2,607	352	7.4	2,484	433	5.7
[3,4-^{14}C]glucose	496	548	0.9	321	443	0.7
$^{14}CO_2$	932	1,200	0.8	492	621	0.8

The amount of acetate formed by reduction of CO_2 was calculated from the radioactivity of acetate and butyrate and the specific activity of CO_2. Butyrate radioactivity is assumed to have arisen by condensation of acetyl units, most likely through exchange reactions between free acetate and the acetyl-*S* coenzyme A used to form butyrate. For subject 1, 167 μmol of the total of 398 μmol of acetate produced was formed from CO_2.

Calculations based on the radioactivity of the methyl carbon of acetate formed from the C-1 position by subject 1 indicated that 111 μmol was produced from the C-1 and C-2 positions by the EMP pathway. Since the EMP pathway yields an equivalent amount of acetate from the C-6 and C-5 positions, the total derived from the methyl and carbonyl carbons of the pyruvate formed from glucose is 222 μmol. This amount, plus the 167 μmol formed from CO_2, gives a total of 389 μmol of acetate. The value calculated from radioisotope data is 98% of the amount of acetate measured by HPLC analysis. Similar calculations for subject 2 indicate that 74 μmol of acetate was formed from CO_2 and 158 μmol was formed from the C-1,2 and C-5,6 positions by the EMP pathway, for a total of 232 μmol. This is 85% of the amount determined by HPLC analysis.

***Bifidobacterium* pathway.** The *Bifidobacterium* pathway may contribute to the fermentation of glucose by suspensions from subject 2. Three moles of acetate and 2 mol of lactate is produced per 2 mol of glucose by the *Bifidobacterium* pathway (15). The methyl and carboxyl of two of the three acetates arise from the C-1 and C-2 of glucose, respectively. Both carbons of one of the three acetates are formed from carbon 3 of glucose. More radioactivity was found in the acetate and butyrate produced from [3,4-^{14}C]glucose by suspensions from subject 2 than could be accounted for by the specific activity of CO_2. This suggests that some radioactive acetate was formed from the C-3 of glucose by the *Bifidobacterium* pathway. With this assumption, calculations indicated that the contributions to acetate formation were as follows: *Bifidobacterium*, 21%; CO_2 reduction, 27%; and acetate from the top and bottom two C atoms of glucose via EMP metabolism, 47%. With subject 1, the radioactivity in acetate and butyrate obtained from [3,4-^{14}C]glucose in excess of the amount expected from CO_2 accounted for a maximum of only 6% of the total acetate, based

TABLE 4. Radioactivity of propionate and butyrate

^{14}C precursor	Radioactivity (dpm/μmol)			
	Subject 1		Subject 2	
	Propionate	Butyrate	Propionate	Butyrate
[1-^{14}C]glucose	4,245	8,179	2,885	6,478
[3,4-^{14}C]glucose	2,223	2,010	1,340	1,109
$^{14}CO_2$	3,703	5,052	3,390	1,827

TABLE 5. Products from cabbage cellulose and $^{14}CO_2$[a]

Product	Total μmol formed	% of total SCFA	Radioactivity (dpm/μmol)
Acetate	216	66	941
Propionate	78	24	2,763
Butyrate	35	11	1,540
CO_2			2,570

[a] Purified cabbage cellulose (50 mg) was added. Hydrogen did not accumulate, and methane was not formed.

on the assumption of a *Bifidobacterium* contribution to the overall fermentation. More rigorous evaluation of the contribution of the *Bifidobacterium* pathway to the overall fermentation can be accomplished by studies of fermentation of ^{13}C-labeled glucose and nuclear magnetic resonance (NMR) analysis of products.

Radioactivity of propionate. Table 4 shows the specific radioactivity of propionate obtained after incubation with the different radioactive precursors. Suspensions from both subjects incorporated ^{14}C from all three of the labeled precursors into propionate. The incorporation of $^{14}CO_2$ can occur by formation of oxalacetate and its subsequent conversion through a four-carbon pathway to propionate (5), by a pyruvate-CO_2 exchange reaction (22), or by both mechanisms. The maximum specific radioactivity possible if all carboxyls of propionate are labeled by CO_2 fixation and exchange is equal to the specific radioactivity of CO_2. The specific activities of propionate from subjects 1 and 2 were 1.09 and 0.96 times that of CO_2, respectively. This indicates that propionate is labeled by both CO_2 fixation and CO_2 exchange reactions.

The labeling of propionate by [1-^{14}C]glucose fermented by the EMP pathway leads to labeling of the 2 or 3 position of propionate. From the expected labeling of those positions by [1-^{14}C]glucose and exchange of the carboxyl group, the ratios of observed to expected radioactivity were 1.0 and 0.74 for subjects 1 and 2, respectively. Definitive resolution of the labeling of propionate requires separate measurements of the labeling of individual carbon atoms, which can be accomplished by using ^{13}C-labeled precursors and product analysis by NMR spectroscopy.

Radioactivity of butyrate. Table 4 shows the specific radioactivity of butyrate obtained after incubation with the different radioactive precursors. The labeling of butyrate by C-1 of glucose (Table 4) is consistent with formation of acetyl-*S* coenzyme A from pyruvate generated by the EMP pathway. Acetate formed from added $^{14}CO_2$ or CO_2 produced from the 3 and 4 carbons of glucose is also incorporated into butyrate (Table 4). Free radioactive acetate appears to be easily incorporated into acetyl-*S* coenzyme A and then into butyrate. The specific radioactivity of butyrate when the suspensions from subject 1 were incubated with $^{14}CO_2$ was 2.4 times the specific radioactivity of acetate and 1.9 times that of acetate when [3,4-^{14}C]glucose was used. The corresponding ratios for subject 2 were 1.6 and 1.5, respectively. The theoretical maximum ratio is 2, and the ratio of 2.4 is probably due to the inaccuracies of the methods used.

Fermentation of cabbage cellulose fraction. We examined the fermentation of a purified cabbage cellulose fraction by a fecal suspension from subject 1 in the presence of $^{14}CO_2$. Table 5 shows the products formed and their specific radioactivities. The distribution of radioactivity in the acetate methyl (43%) and carboxyl (57%) carbons indicated that acetate was formed by CO_2 reduction during cellulose fermentation. The amount of acetate formed from CO_2 was 23% of the total acetate. The specific radioactivity of propionate was essentially the same as that of CO_2, indicating that both carboxylation and exchange reactions labeled the carboxyl group. Since the specific radioactivity of butyrate was 1.6 times that of acetate, significant amounts of free acetate exchanged with the acetyl units of butyrate. The results indicate that the fermentation of purified cabbage cellulose by subject 1 follows the same pathways as are used for glucose fermentation.

Overall pathways. The results and calculations are consistent with operation of the EMP pathway as the major colonic pathway for catabolism of glucose and the formation of significant amounts of acetate by the Wood-Ljungdahl pathway. The overall fermentation in the bovine rumen also proceeds mainly via the EMP pathway (14). Lajoie et al. (7) showed that the colonic microflora reduced $^{13}CO_2$ to acetate with molecular H_2 as the electron donor. Other studies indicate that reduction of CO_2 to CH_4 is a major component of the overall fermentation in only 10 to 30% of all adult humans (17). The results of this study are the first demonstration of the importance of the Wood-Ljungdahl pathway in the formation of acetate during glucose fermentation by the human colonic microflora. The contribution of the Wood-Ljungdahl pathway is large. CO_2 reduction accounts for about one-third of the acetate formed by suspensions from subject 1 and about one-fourth of the acetate formed by suspensions from subject 2. CO_2 reduction is probably associated with the utilization of free H_2 formed as an intermediate fermentation product and the utilization of electrons by homoacetogenic, glucose-fermenting species (23). The other major sources of acetate are produced from the glucose carbon skeleton, probably by a combination of the homoacetate and propionate fermentations. There may be a significant contribution of the *Bifidobacterium* pathway in some individuals. Propionate is formed by CO_2 fixation pathways, as in the propionibacteria, and butyrate is formed by classical routes of acetyl-*S* coenzyme A condensation.

More investigations are necessary to establish how general these pathways are in different individuals and the influence of age, diet, and diseases of the large bowel. Individuals in whom large amounts of methane are formed in the colon might be expected to have diminished contributions of the Wood-Ljungdahl pathway, because reduction of CO_2 to methane would compete with formation of acetate from CO_2. Changes in *Bifidobacterium* concentrations that are related to diet and health (10) or colonization of the neonate colon (1) would alter the patterns of cleavage of the glucose carbon skeleton.

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