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Glucose Fermentation Products of *Ruminococcus albus* Grown in Continuous Culture with *Vibrio succinogenes*: Changes Caused by Interspecies Transfer of H_2

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The influence of a H_2-utilizing organism, *Vibrio succinogenes*, on the fermentation of limiting amounts of glucose by a carbohydrate-fermenting, H_2-producing organism, *Ruminococcus albus*, was studied in continuous cultures. Growth of *V. succinogenes* depended on the production of H_2 from glucose by *R. albus*. *V. succinogenes* used the H_2 produced by *R. albus* to obtain energy for growth by reducing fumarate in the medium. Fumarate was not metabolized by *R. albus* alone. The only products detected in continuous cultures of *R. albus* alone were acetate, ethanol, and H_2. CO_2 was not measured. The only products detected in the mixed cultures were acetate and succinate. No free H_2 was produced. No formate or any other volatile fatty acid, no succinate or other dicarboxylic acids, lactate, alcohols other than ethanol, pyruvate, or other keto-acids, acetoin, or diacetyl were detected in cultures of *R. albus* alone or in mixed cultures. The moles of product per 100 mol of glucose fermented were approximately 69 for ethanol, 74 for acetate, 237 for H_2 for *R. albus* alone and 147 for acetate and 384 for succinate for the mixed culture. Each mole of succinate is equivalent to the production of 1 mol of H_2 by *R. albus*. Thus, in the mixed cultures, ethanol production by *R. albus* is eliminated with a corresponding increase in acetate and H_2 formation. The mixed-culture pattern is consistent with the hypothesis that nicotinamide adenine dinucleotide (reduced form), formed during glycolysis by *R. albus*, is reoxidized during ethanol formation when *R. albus* is grown alone and is reoxidized by conversion to nicotinamide adenine dinucleotide and H_2 when *R. albus* is grown with *V. succinogenes*. The ecological significance of this interspecies transfer of H_2 gas and the theoretical basis for its causing changes in fermentation patterns of *R. albus* are discussed.

Fermentation products, particularly ethanol and lactate, of pure cultures of a number of important rumen bacteria are not usually formed nor are they usually significant intermediates in the rumen fermentation (7). The major products of the fermentation of ingested food, mainly plant carbohydrates, by the mixed rumen microbial population are acetic, propionic, and butyric acids, and the gases CH_4 and CO_2 (7). Hungate (7) suggested that, in the rumen, the electrons derived from the oxidation of fermentation substrates by carbohydrate-fermenting, ethanol- or lactate-producing rumen microorganisms are somehow shunted away from the reduction of the fermentation intermediate, pyruvate, or electron acceptors derived from pyruvate. He proposed that these electrons are available for use by H_2-utilizing, methane-forming bacteria in the ecosystem. In pure culture, however, these same carbohydrate-fermenting organisms have to supply their own electron sink in the form of a lactate- or ethanol-forming system in order to satisfy the oxidation-reduction balance requirements of any anaerobic fermentation.

We began a series of experiments to attempt to test Hungate's hypothesis. In brief, our goal was to compare the fermentation products produced by the rumen bacterium, *Ruminococcus albus*, in pure culture with those produced by *R.*

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albus in mixed culture with a H_2-utilizing bacterium. *R. albus* is a cellulolytic bacterium that was shown to produce ethanol, acetate, formate, H_2, and CO_2 (some strains produce small amounts of succinate and lactate) from cellobiose (1, 9). We planned to mix *R. albus* with *Vibrio succinogenes*, an organism that can obtain energy for growth by coupling the oxidation of H_2 or formate with the reduction of fumarate to succinate. If Hungate's hypothesis is correct, it would be expected that the only products of the mixed-culture fermentation of carbohydrate would be acetate, succinate (from fumarate reduction by the vibrio), and CO_2. We decided to use a continuous-culture system for these experiments. Although there were several reasons for this decision, a major reason was the belief that we could synchronize the growth of one species with respect to the other better in continuous than in batch culture.

Prior to the mixed-culture experiment, it was essential to accurately establish a fermentation balance for *R. albus* in continuous culture. Hungate studied the growth of *R. albus* in continuous culture, but he did not characterize the fermentation products (6). Fermentation product analyses for *R. albus* were done in batch cultures with cellobiose as the substrate (1, 9), but these were not complete balances. In addition, there is no guarantee that batch and continuous-culture fermentations will be the same. An anaerobic continuous culture apparatus was designed which permits the cultivation of nonsporeforming anaerobes such as *R. albus* and also permits gas accumulation and measurement for complete product analysis (8). We used this apparatus for the determination of a fermentation balance for *R. albus* in a glucose-limited continuous culture at various growth rates and for similar experiments with mixed cultures of *R. albus* and *V. succinogenes*. In the latter case, H_2 production by *R. albus* and fumarate in the medium supported the growth of *V. succinogenes*. *V. succinogenes* can obtain energy for growth by reducing fumarate with H_2, but it will not use glucose, ethanol, or acetate as energy sources in the presence or absence of fumarate (15). We will show that *R. albus* ferments glucose to ethanol, acetate, hydrogen, probably CO_2, and unidentified products when glucose limits growth in a chemostat. No formate is produced. In mixed culture, all of the H_2 produced by *R. albus* is used by *V. succinogenes* to reduce fumarate to succinate. In addition, no ethanol is produced in the mixed-culture system, but acetate production increases relative to the monoculture fermentation of *R. albus*. The increased acetate carbon corresponds to the decrease in ethanol carbon relative to the monoculture, and the electrons found in ethanol in the monoculture are diverted to succinate formation by the vibrio presumably by interspecies transfer of H_2.

MATERIALS AND METHODS

Organism, media, and growth conditions. *R. albus* strain 7 was used. It was grown in continuous culture in a medium of the following composition (per liter): KH_2PO_4, 0.48 g; K_2HPO_4, 0.48 g; $(NH_4)_2SO_4$, 0.48 g; NaCl, 0.96 g; Trypticase, 5.0 g; yeast extract, 1.0 g; isobutyric acid, isovaleric acid, and DL-2-methylbutyric acid, 0.1 ml of each; cysteine·hydrochloride, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.13 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; Na_2CO_3, 4.0 g; sodium fumarate, 3.9 g; glucose, 1.0 g, resazurin, 1.0 mg. The fumarate, cysteine, carbonate, fatty acids (neutralized with NaOH), glucose, magnesium sulfate, and calcium chloride were each made up as separate solutions, autoclaved, cooled, and individually added to the other ingredients which were autoclaved as a single solution (13.5 liters) in a 19-liter carboy. The total volume of the medium was 15 liters; the final pH was 6.7 to 6.8 after saturation with 100% CO_2. Batch cultures for maintenance and inoculation of the continuous-culture vessel were grown in this same medium with or without fumarate.

The continuous-culture apparatus and operating procedures are described in detail in a separate publication (8). In brief, the culture vessel held 345 ml of medium up to an overflow tube. The entire system was initially made anaerobic by flushing with O_2-free CO_2. After inoculation, gas flow through the culture and collecting vessels was discontinued, and the growth and collection portion of the apparatus were maintained as a closed, anaerobic system. Medium was pumped into the culture vessel from the anaerobic reservoir after the inoculum grew up as a batch culture. Produced gases collected within the culture and collection vessels, and pressure build-up was prevented by allowing gases to expand into a collapsed football bladder. The culture overflow was collected in a flask, held at 0 C, that was emptied by applying a positive CO_2 pressure to the system after approximately 2,000 ml of culture was collected. The continuous-culture system was never in a steady state with respect to gas atmosphere composition because of the production of H_2 by *R. albus*. After collection of the overflow, there was no H_2 in the atmosphere. The H_2 concentration increased with time between emptyings of the collection flask.

Continuous cultures were checked periodically for contamination by microscopic examination and inoculation into trypticase soy broth (Baltimore Biological Laboratories) with incubation in air without shaking. *R. albus* does not grow in trypticase soy broth aerobically. A positive check for *R. albus* was carried out by inoculation into the continuous-culture medium with cellulose or xylan substituted for glucose and verifying the ability of the organism to grow on these energy sources.

Fermentation analyses. After emptying the col-

lection flask contents, cells were immediately removed by centrifugation at 13,200 × g for 30 min at 0 C in a Servall RC2B centrifuge. The supernatant solutions were frozen and kept at −20 C until analyzed.

Glucose was determined by the colorimetric glucose oxidase procedure of the Sigma Chemical Co., except that the reagent was dissolved in 0.1 M potassium phosphate buffer, pH 7.0.

Organic acids were initially determined by the method of Ramsey (10). After finding that the only organic acid produced was acetic acid, the method was modified for a rapid batch separation of acetic, formic, fumaric, and succinic acids. Formic acid was of concern because of its reported production in batch cultures (1, 9), and fumaric and succinic acids were of interest because the reduction of fumaric to succinic acid by *V. succinogenes* was used to remove H_2 from the system. All solvents were saturated with 0.5 N H_2SO_4. A 10-g amount of silicic acid was mixed with 0.5 NH_2SO_4 until a free-flowing powder was formed. This was slurried with chloroform and poured into a 12-mm glass chromatographic column plugged with glass wool. The silicic acid was packed under 2 to 3 psi of N_2. A 1-ml amount of the sample was pipetted into a 30-ml beaker and mixed with a few drops of 50% (v/v) H_2SO_4 and 1.6 g of silicic acid until a free-flowing powder was formed. This was poured on top of the silicic acid column through a small layer of chloroform. Finally, a piece of glass wool was added. The branched, volatile fatty acids added to the continuous culture medium were eluted with 80 ml of chloroform, acetic acid with 80 ml of 2% *t*-butanol in chloroform, followed by 140 ml of the same solvent to remove formic acid, fumaric acid with 125 ml of 6% *t*-butanol in chloroform, and succinic acid with 175 ml of 10% *t*-butanol in chloroform. The eluted organic acids were quantitated by titration with 0.01 N ethanolic potassium hydroxide while bubbling a stream of nitrogen through the sample. Solvent blank values were subtracted from the sample values.

Volatile fatty acids (except formate) were also determined by gas chromatography. A 2-ml amount of culture supernatant fluid was acidified with 0.4 ml of 2 N HCl. This solution was directly analyzed on a 6′ × ⅛″ stainless-steel column (6 ft by ⅛ inch [approx. 1.8 m by 0.32 cm]) containing 60% FFAP (5) on 80- to 100-mesh Porapack T. The instrument was a Beckman GC 5 gas chromatograph (Beckman Instruments, Inc.) with a column temperature of 190 C, a helium carrier gas flow rate of 120 ml/min, and a hydrogen flame detector. Volatile fatty acids were identified and quantified by comparison of retention times and peak heights with those of known standards.

Alcohols were determined by gas chromatography of the acidified solutions used for gas chromatographic analysis of volatile fatty acids. The procedures used were identical to those used for gas chromatographic analysis of volatile fatty acids except for a reduction of the column T to 120 C.

Gas analysis was by gas chromatography. Details of gas sampling, volume measurements, and gas chromatographic procedures are given in a separate publication (8). It was necessary to sample from different sites in the culture-collection system to obtain H_2-production values because of poor mixing of H_2 in the gas atmosphere (8).

Qualitative analysis for acetoin or diacetyl (or both) was by the Voges-Proskauer reaction. A 0.6-ml amount of 5% α-naphthol (in 40% KOH) was added to 1 ml of culture supernatant fluid, and the mixture was incubated at 25 C for 1 h with intermittent vigorous shaking.

Incorporation of [U-^{14}C]glucose into cells. [*U*-^{14}*C*]glucose (190 mCi/mmol) was injected into the culture vessel as described in the previous publication (8). A rubber tube between the overflow side arm and the collection flask was clamped to collect a small volume of culture overflow. After removing a sample with a hypodermic needle and syringe for analysis at a particular time, the clamp was removed to clear the overflow tube. The clamp was replaced, and the process was repeated for the next sampling period. The sample was immediately cooled in a mixture of ice and salt, and 1 ml was centrifuged at 27,000 × g at 0 C for 15 min in a Sorvall RC2B centrifuge. Portions of the sample before centrifugation and the supernatant fluid after centrifugation were transferred to scintillation fluid which contained (per liter of dioxane): naphthalene, 100 g; Cab-O-Sil (Beckman Instruments, Inc.), 20 g; and PPO, 5 g. Details of the method of calculation of ^{14}C incorporation into cells are given below.

RESULTS

Incorporation of glucose into cells. In order to determine fermentation balances for *R. albus* and the mixed cultures, it was necessary to measure the amount of glucose incorporated into cells. This was accomplished by determining the incorporation of [*U*-^{14}*C*]glucose into cells from cultures of *R. albus* alone and cells from the mixed culture. If labeled glucose were added to a usual culture system in which the saturation constant for glucose uptake is very small, the label would be rapidly taken up by the cells and incorporated into the cells or degraded to end products. The counts found in the cells would then decrease with a rate constant equal to the dilution rate. A significant amount of glucose, however, remained in the supernatant fluids, especially at faster dilution rates. This is indicative of a high saturation constant (K_s) for glucose metabolism by *R. albus* (14). Because of the high saturation constant, complete metabolism of labeled glucose was not achieved until a significant time after the addition of label (Fig. 1 and 2). The percentage of glucose incorporated into cells was determined by comparing the counts per minute found in the cells to the total counts per minute in cells per supernatant fluid, i.e., nonmetabolized [^{14}C]glucose plus fermentation products, at the time of sampling.

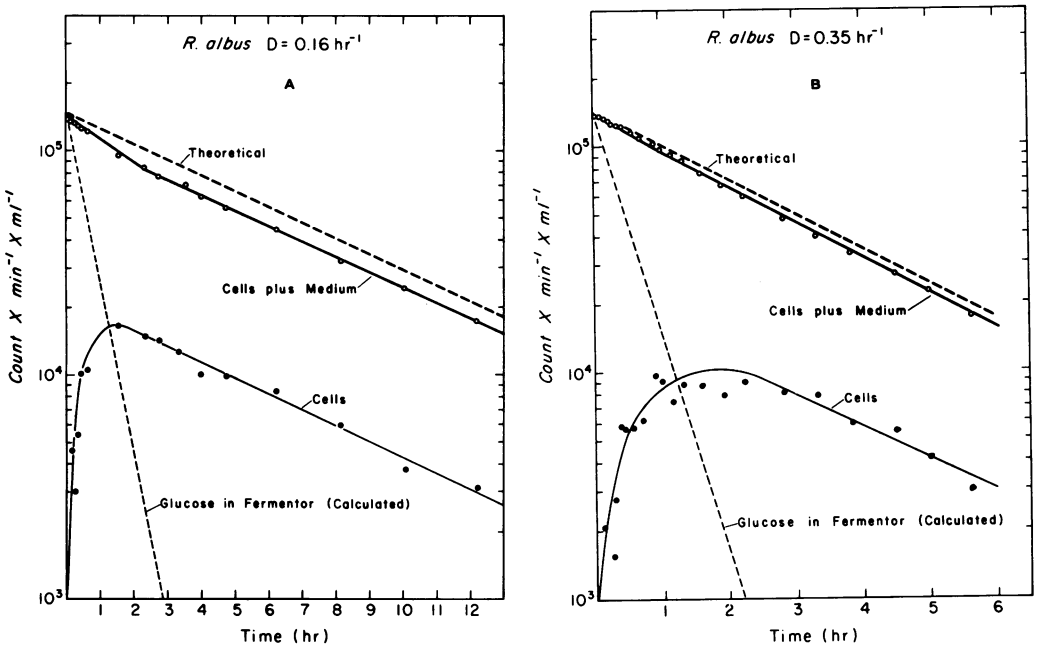

FIG. 1. *Incorporation of [^{14}C]glucose into R. albus cells. Symbols: Theoretical washout (no loss of ^{14}C) (- - -); ^{14}C in liquid portion (cells included) (-O-); ^{14}C in cells (-●-●); a calculated curve for disappearance of [^{14}C] glucose from the fermentation vessel (- - -). A, Dilution rate 0.16/h; B, dilution rate 0.35/h.*

The experimental total counts per minute at the sampling times do not include all products because of loss of $^{14}CO_2$ from the medium. In order to calculate percent incorporation, it was necessary to calculate the theoretically expected total counts for the times of sampling.

The theoretical (no loss of ^{14}C) total counts per minute expected at time t were calculated by first extrapolating the total counts per minute in the initial samples back to time zero (t_0). Separate experiments showed that the extrapolated t_0 values were the same as the t_0 values obtained by diluting the ^{14}C label to the same extent as the label actually added to the fermentors. The theoretical counts per minute at time t were then calculated from the equation: $\ln (x/x_0) = -D(t)$, where x_0 is the extrapolated counts per minute at t_0, x is the calculated counts per minute at t, and D is the dilution rate. Figures 1A and 1B show the calculated theoretical washout of total label from the *R. albus* fermentors at dilution rates of 0.16 and 0.35/h along with the actual washout of label in the liquid portion (cells included), and the amount of label incorporated into the cells. Figures 2A and 2B show analogous data for the mixed culture at dilution rates of 0.12 and 0.37/h. Figures 1 and 2 also show calculated curves for the rate of disappearance of [^{14}C]glucose from the fermentor. It was useful to calculate these values to determine when essentially all of the labeled glucose was converted to fermentation products or cells. The subsequent rate constants for washout of label in cells should then be identical to the calculated rate constants for washout of total label. Calculation of the percent incorporation into cells from measured values obtained after labeled substrate is completely used eliminates errors that may be introduced when sampling is carried out when cells are actively metabolizing the labeled substrate. Since labeled glucose disappears from the culture vessels as a function of washout and metabolism, the decrease in concentration can be evaluated from the following equations:

$$-dS_c/dt = D(S_r - S)(S_c) + D(S_c) \quad (1)$$

where S_c is the [^{14}C]glucose concentration in the fermentor, S_r is the glucose concentration in the reservoir, and S is the steady-state glucose concentration in the fermentor. $D(S_r - S)$ is the rate constant for metabolism of [^{14}C]glucose because it is the rate of metabolism of glucose in the fermentor. $D(S_c)$ evaluates disappearance of [^{14}C]glucose due to washout. Rearrangement gives,

$$-dS_c/dt = D[(S_r - S) + 1]S_c \quad (2)$$

$D[(S_r - S) + 1]$ is a rate constant (K_t) for the

disappearance of added [^{14}C]glucose from a particular fermentor. K_f can be evaluated at a particular D by determining S, and equation 2 is equal to

$$-dS_c/dt = K_f S_c \quad (3)$$

Integration of (3) gives

$$S_c = 1/A_e^{k_f t}$$

Equation 3 was used to calculate the values shown in Fig. 1 and 2 for [^{14}C]glucose disappearance from the fermentors. The values for S used to calculate the respective K_f values were from determinations of glucose in culture supernatant fluids. The label in cells reaches a peak at approximately the time that the labeled glucose in the fermentor is less than 5% of the initial label. Subsequent to the attainment of the peak, cell label washes out with the same rate constant as the theoretical or actual label in the effluent from the culture vessel. Since the theoretical value is the theoretical total label in the effluent at any time t, the decreasing cell label at the points of constant, negative slope divided by the theoretical total value gives the proportion of label incorporated into the cells. The slopes for the washout of labeled cells (Fig. 1 and 2) subsequent to the point at which labeled glucose in the culture vessel was calculated to be 1% of the original label were calculated by the method of least squares. Table 1 shows the calculated slopes, the slopes for theoretical washout, and the calculated percent incorporation into cells for each fermentor. Since the values for *R. albus* alone were not significantly different, the value of 15% was used to calculate percent incorporation of glucose into cells in all subsequent experiments. For the mixed cultures, we used 21% incorporation into cells for dilution rates of 0.2/h or less and 25% for dilution rates of 0.3/h or more.

Analysis of R. albus fermentation end products. A sample from a culture (dilution rate of 0.35/h) was analyzed with the complete solvent system of Ramsey with fraction collection. The only acid produced was acetic acid. No propionic or longer-carbon-chain volatile acid, lactic, formic, pyruvic, or succinic acids were produced. No keto acid or neutral ketone, such as acetone, could be detected as the hydrazone. Gas chromatographic analysis of volatile fatty acids showed that acetic acid was the only volatile acid produced. No alcohols other than ethanol could be found by gas chromatographic analysis. The sample was Voges-Proskauer negative. The methods utilized would detect most common microbial fermentation end products except glycerol.

Samples from cultures run at different dilu-

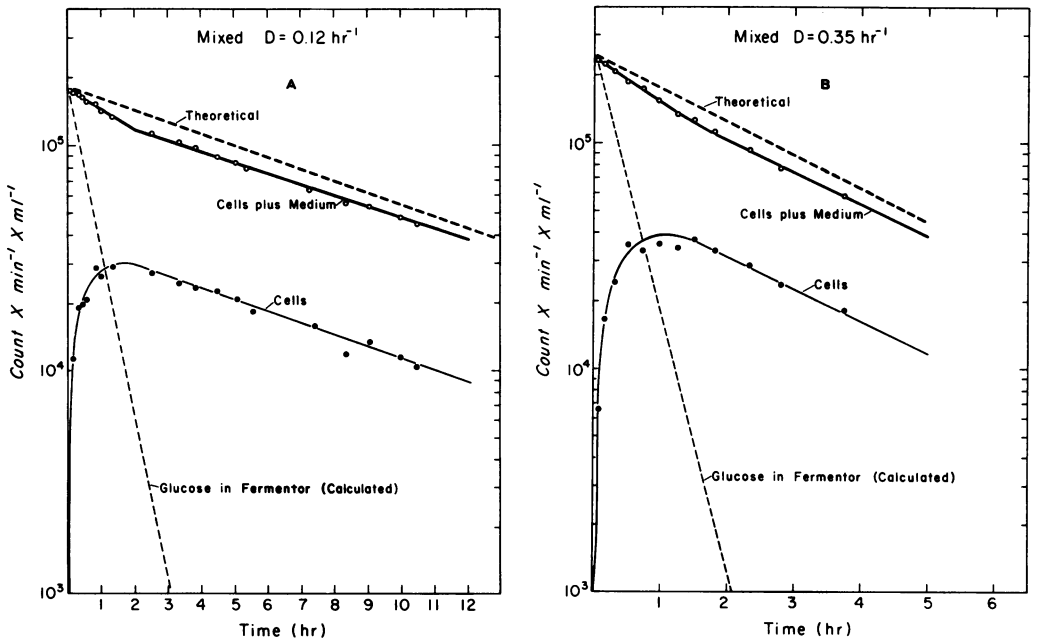

FIG. 2. *Incorporation of* [^{14}C]glucose into cells. Symbols: Theoretical washout (no loss of ^{14}C) (- - -); ^{14}C *in liquid portion (cells included)* (-O-); ^{14}C *in cells* (-●-); *calculated curve for disappearance of* [^{14}C]*glucose from the fermentation vessel* (- - -). *A, Dilution rate 0.12/h; B, dilution rate 0.35/h.*

TABLE 1. *Calculation of percent of [U-^{14}C]glucose incorporated into cells*

Experimental values	Dilution rate/h			
	R. albus		*R. albus* + *V. succinogenes*	
	0.16	0.35	0.12	0.35
Washout slopes (log counts per min per h)				
Theoretical total radioactivity	0.069	0.152	0.052	0.152
Cell radioactivity	0.070	0.141	0.053	0.142
10^4 counts per min per ml at y intercept				
Theoretical total radioactivity	14.45	14.15	18.10	24.20
Cell radioactivity	2.13	2.07	3.81	5.98
Glucose incorporated into cells (%)	14.7	14.6	21.0	24.7

tion rates were then analyzed for acids with the Ramsey column modified for batch collection, for ethanol by gas chromatography, for glucose with glucose oxidase, and for H_2 by volume measurements and gas chromatography. Table 2 shows the data collected for all analyses at all dilution rates. The acetate values in Table 2 are the gas chromatography values. The titration values obtained from the modified Ramsey procedure were slightly higher but were considered to be less accurate because of the poor titration end point obtained with the low concentrations of acetate in large volumes of solvent. Formate, lactate, or succinate production were not detected in any of the samples by the modified Ramsey procedure. The glucose values in Table 2 are corrected for incorporation of glucose into cells. Table 3 shows the same product data expressed as moles of product per 100 mol of glucose along with the means and standard deviations. The CO_2 values were calculated by assuming that the moles of CO_2 produced were equal to the sum of the moles of ethanol and acetate produced. Carbon recoveries and the oxidized products/reduced products (O/R) index, calculated from the means, were 72% and 0.76, respectively.

As can be seen in Table 3, the data are scattered. These are most likely due to inherent experimental difficulties. H_2 does not equilibrate in the gas phase of the fermentation apparatus, and it is necessary to use gas samples from several points in the apparatus for gas chromatography to obtain best values for the H_2 concentration in a particular portion of the gas phase in the system (8). The commercial Trypticase in the medium is contaminated with acetate at a level which gives a blank concentration of 5.6 to 6.5 mM in the medium, or about 1 to 4 times the concentrations of acetate produced in the culture vessels.

Analysis of mixed-culture fermentation products. Separate batch-culture experiments showed that *V. succinogenes* did not grow on the medium used in the chemostat unless *R. albus* was present. The concentration of fumarate in the medium remains unchanged in batch or continuous monocultures of *R. albus*. Examination of selected, continuous, mixed cultures by gas chromatography and liquid chromatography on silicic acid with the Ramsey column showed that acetic and succinic acids were the only acid products. Gas chromatographic analysis showed that no alcohols, including ethanol, propanol, isopropanol, or butanol were produced in the mixed culture. Acetoin or diacetyl were not produced. No H_2 ever accumulated in the mixed-culture system.

Samples from cultures run at different dilution rates were then analyzed for acids with the Ramsey column modified for batch collection, for ethanol and acetate by gas chromatography, and for glucose with glucose oxidase. Table 4 shows the data collected for all analyses at all

TABLE 2. *Glucose fermented and products produced by R. albus at different dilution rates*[a]

Dilution rate (h)	Glucose fermented[b,c]	Products			
		Ethanol[c]	Acetate[c]	H_2[c]	H_2[d]
0.17	3.9	3.2	2.7	9.7	.21
0.17	4.1	3.4	2.7	8.6	.18
0.25	4.2	2.6	4.9	11.1	.21
0.25	4.1	3.4	3.7	7.2	.15
0.25	3.5	2.5	2.3	9.6	.19
0.33	3.0	2.2	2.3	9.2	.19
0.33	3.4	2.0	2.3	9.4	.20
0.49	3.5	2.0	2.1	5.9	.14
0.59	2.7	1.4	1.9	4.6	.07
0.59	2.7	1.8	1.6	7.2	.16

[a] An amount of medium equivalent to approximately four to five times the fermentor volume (345 ml) was pumped through the fermentor and removed from the collection flask at each dilution rate; approximately 2.5 liters was collected for analysis at each dilution rate. The time for collecting 2 liters ranged from approximately 64 h for dilution rate 0.09/h to 12 h for dilution rate 0.59/h.

[b] Glucose fermented was calculated by correcting the difference between the medium reservoir and culture supernatant fluids for the amount incorporated into cells.

[c] Micromoles used or produced per milliliter of culture.

[d] Partial pressure at time of sampling in atmosphere.

TABLE 3. *Products produced by R. albus per 100 mol glucose*

Dilution rate (h)	Ethanol	Products (mol/100 mol of glucose)		
		Acetate	H_2	CO_2[a]
0.17	82	69	249	151
0.17	83	66	211	149
0.25	62	117	266	179
0.25	83	90	176	173
0.25	71	67	275	138
0.33	74	76	310	150
0.33	59	66	278	125
0.49	57	60	170	117
0.59	52	71	172	123
0.59	65	59	264	124
Mean	69	74	237	143
SD[b]	11	17	51	21
SE[c]	4	5	16	7

[a] Calculated as equal to ethanol plus acetate.
[b] Standard deviation.
[c] Standard error.

TABLE 4. *Glucose fermented, fumarate used, and products produced at different dilution rates in mixed cultures*[a]

Dilution rate (h)	Glucose fermented (mM)	Fumarate used (mM)	Products (mM)	
			Acetate	Succinate (H_2)[b]
0.11	4.4	18.8	6.2	16.6
0.12	4.4	18.7	6.2	15.9
0.19	3.9	18.5	6.0	15.6
0.19	4.0	18.2	5.5	15.6
0.33	3.3	13.9	5.3	12.7
0.35	3.3	15.6	4.5	15.2
0.65	1.2	4.8	2.0	4.6
0.65	1.3	5.0	2.0	4.2

[a] See Table 2, footnotes a and b, for experimental details.
[b] H_2 is considered to be equivalent to the amount of succinate produced.

dilution rates. The glucose values are corrected for incorporation of glucose into cells. Table 5 shows the same product data expressed as moles of product per 100 mol of glucose fermented along with the means and standard deviations. Succinate is considered to be equivalent to the number of moles of H_2 produced by *R. albus* and used by *V. succinogenes* (H_2 + fumarate → succinate). The CO_2 values were calculated by assuming that the moles of CO_2 produced were equal to the moles of acetate produced. Carbon recoveries and the oxidized/reduced products (O/R) index, calculated from the means were 74% and 0.77, respectively.

Acetate incorporation into cells. One possible reason for the low carbon recovery and excess of reduced products could be the net uptake of significant amounts of acetate, present in the uninoculated medium, into cell material. If the missing carbon is calculated as acetate and equivalent CO_2, the O/R balance becomes 1.04. We examined the amount of [^{14}C]acetate incorporated into cell material in a batch culture of *R. albus* grown with 5.4 mM glucose, in the same medium used in the chemostat experiments. The [^{14}C]acetate (12.3 mCi/mmol) was added to the uninoculated medium (50 nCi/ml) which contained 5.3 mM acetate. The medium acetate was a contaminant in the commercial Trypticase and was between 5.5 and 6.5 mM in all of the media used in these studies. When 5.5 mM glucose was used, the final level of acetate in the medium was 8.7 mM and the cells had incorporated 5% of the [^{14}C]acetate. Under these same conditions, the cells incorporated 9% of added [^{14}C]glucose. Assuming a similar relationship between glucose and acetate incorporation in the chemostat cultures, the total acetate values could be low by about 8.3% because of the approximately 15% incorporation of glucose into cells in the chemostat. For the mean values of Table 4, this acetate and equivalent CO_2 would account for approximately 4% of the missing carbon. Although an acetate-incorporation experiment was not done with a mixed culture, the similarities between C recoveries and O/R indices for single and mixed continuous cultures indicate that it is unlikely that acetate incorporation can account for a significant amount of the missing C in either case.

TABLE 5. *Fumarate used and products produced per 100 mol of glucose in mixed cultures*

Dilution rate (h)	Fumarate used	Products (mol × 100 mol of glucose)		
		Acetate	Succinate (H_2)	CO_2[a]
0.11	426	139	376	139
0.12	423	139	359	139
0.19	473	153	398	153
0.19	457	137	391	137
0.33	419	158	383	158
0.35	471	137	459	137
0.65	406	168	389	168
0.65	373	147	313	147
Mean	431	147	384	147
SD	34	11	41	11
SE	12	4	20	4

[a] Calculated as equal to acetate.

DISCUSSION

Table 6 shows a comparison of the fermentation balance data of the monoculture of *R. albus* with the data for the mixed *R. albus-V. succinogenes* system. Although ethanol is a major product of the *R. albus* monoculture fermentation, ethanol is not produced in the mixed system. Instead, acetate production increased in the mixed system by an amount almost equivalent to the amount of ethanol produced by the monoculture of *R. albus*. The amount of succinate produced (H_2 equivalents) indicates an increase in H_2 production in an amount almost equivalent to the electron deficit caused by the disappearance of ethanol and the increase in acetate in the monoculture to mixed culture change. These results essentially prove the Hungate hypothesis (7), i.e., a H_2-utilizing organism can cause electrons to be shifted away from the production of a typical fermentation product, e.g., ethanol, to give the more oxidized product, acetate. The shifted electrons probably are converted to H_2 (see below) and used by the H_2-utilizer for reduction of its oxidized substrate. Thus, the explanation offered by Hungate (7) for the usual lack of production or intermediate formation of ethanol in the rumen ecosystem appears to be correct, although, in the rumen ecosystem, methane-forming bacteria would be the major H_2-utilizing organisms. It is likely that the same explanation offered by Hungate (7) for the usual lack of production or intermediate formation of lactate in the rumen is also correct.

TABLE 6. *Comparison of R. albus monoculture fermentation with R. albus-V. succinogenes mixed fermentation*

Substrate used and products	(Mol × 100 mol of glucose)			
	R. albus		*R. albus* plus *V. succinogenes*	
	Uncorrected	Corrected[a]	Uncorrected	Corrected[a]
Substrate				
Fumarate	0	0	431	431
Products				
Ethanol	69	69	0	0
Acetate	74	131	147	200
H_2[b]	237	237	384	384
CO_2[c]	143	200	147	200
C recovery (%)	73	100	74	100
O/R	0.76	1.09	0.77	1.04

[a] Assuming that the missing carbon is acetate plus CO_2.

[b] Succinate equivalents in the case of the mixed culture.

[c] Calculated as equal to 2 carbon compounds.

Although the lack of complete carbon recovery in the fermentation balances cannot be explained on the basis of definitive experimental evidence, it seems highly unlikely that the missing carbon can be implicated in the fermentation product shift that occurs in the transition from monoculture to mixed culture. It would have to be argued that unknown oxidized product, produced in the monoculture, becomes reduced in the mixed culture, at the expense of ethanol production, to an unknown product. This would be inconsistent with the observation that the increased succinate production (H_2 equivalents) in the mixed culture is almost equivalent to the electron deficit caused by ethanol disappearance in the change from monoculture to mixed culture. Since the carbon recovery and O/R index are almost identical in the monoculture and mixed-culture experiments (Table 6), it seems reasonable to assume that *V. succinogenes* does not influence the production of the as yet unidentified product(s) produced by *R. albus*.

We will now discuss the probable biochemical explanation of the shift of electrons from ethanol production in the monoculture to succinate formation in the mixed system. For this purpose, it is convenient to start with the probable fermentation equations for both systems. Table 6 shows the experimental fermentation values and the same values corrected on the assumption that the missing carbon is in the form of acetate and CO_2. As was previously indicated, a compound or compounds equivalent to acetate plus CO_2 would provide the carbon and O/R state necessary to satisfy a balanced equation for glucose fermentation. Table 7 shows likely partial reactions for the monoculture and mixed-culture fermentations, based on the corrected values of Table 6, and the sums of the partial reactions. Although most of the reactions have not been directly demonstrated in *R. albus*, they represent common bacterial enzyme reactions. The most unique aspect of the partial sequence is the postulation of a nicotinamide adenine dinucleotide (reduced form) (NADH)-linked hydrogenase in *R. albus*. The presence of the hydrogenase would permit two alternative electron pathways for the reoxidation of the NADH generated by oxidation of glyceraldehyde-3-phosphate (G3P). Either acetyl-coenzyme A could be reduced, through acetaldehyde, to ethanol, or the NADH could be directly oxidized to H_2 and nicotinamide adenine dinucleotide (NAD). The free energy change at pH 7 and 25 C ($\Delta G'_0$) for

TABLE 7. *Partial reactions for monoculture and mixed culture fermentations*

Type of culture	Reaction[a]
Monoculture or mixed culture	$100\ C_6H_{12}O_6 + 200\ ATP \rightarrow 200$ glyceraldehyde-3-P(G3P) $+ 200\ ADP$
	200 G3P $+ 200\ NAD^+ + 200$ Pi $\rightarrow 200$ 1,3-diphosphoglyceric acid $+ 200\ NADH + 200\ H^+$
	200 DPGA $+ 400\ ADP \rightarrow 200\ CH_3COCO_2H + 400\ ATP$
	$200\ CH_3COCO_2H + 200$ CoASH $\rightarrow 200\ CH_3COSCoA + 200\ H_2 + 200\ CO_2$
Monoculture	$70\ CH_3COSCoA + 140\ NADH + 140\ H^+ \rightarrow 70\ C_2H_5OH + 140\ NAD^+ + 70$ CoASH
	$130\ CH_3COSCoA + 130\ ADP + 130$ Pi $\rightarrow 130\ CH_3CO_2H + 130\ ATP + 130$ CoASH
	$60\ NADH + 60\ H^+ \rightarrow 60\ H_2 + 60\ NAD^+$
	Sum: $100\ C_6H_{12}O_6 + 330\ ADP + 330$ Pi $\rightarrow 70\ C_2H_5OH + 130\ CH_3CO_2H + 200\ CO_2 + 260\ H_2 + 330\ ATP$
Mixed culture	$200\ CH_3COSCoA + 200\ ADP + 200$ Pi $\rightarrow 200\ CH_3CO_2H + 200\ ATP + 200$ CoASH
	$200\ NADH + 200\ H^+ \rightarrow 200\ H_2 + 200\ NAD^+$
	$400\ H_2 + 400$ fumarate $\rightarrow 400$ succinate
	Sum: $100\ C_6H_{12}O_6 + 400\ ADP + 400$ Pi $+ 400$ fumarate $\rightarrow 200\ CH_3CO_2H + 200\ CO_2 + 400$ succinate $+ 400\ ATP$

[a] Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; G3P, glyceraldehyde-3-phosphate; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; DPGA, diphosphoglyceric acid; CoASH, coenzyme A.

the oxidation of G3P to H_2 and 1,3-diphosphoglyceric acid (DPGA) is +5.83 kcal (3). The equilibrium constant is 5.28×10^{-5}. This energetically unfavorable reaction can be coupled to the exergonic production of ethanol in the monoculture or the exergonic formation of succinate in the mixed system. The formation of H_2 from the conversion of pyruvate to acetate and CO_2 is a thermodynamically favorable reaction, i.e., $\Delta G'_0$ is −13.69 kcal (3). Although the oxidation of G3P to DPGA and H_2 is not a thermodynamically favorable reaction, the balance of intermediate reactions for the monoculture (table 7) suggests that the specific conditions in the cell and its environment permit some flow of electrons from NADH to H_2. The equations of Table 7 suggest that approximately 30% of the NADH generated by G3P oxidation is oxidized to H_2 and NAD. In the mixed cultures, however, it would seem that 100% of the NADH generated in glycolysis is oxidized to H_2, which, in turn, is used for succinate formation.

The situation is analogous to the formation of CH_4 and acetate from ethanol or pyruvate and CO_2 by a mixed-culture system (2, 11–13). In this case, the pulling of the NAD-dependent oxidation of ethanol to acetaldehyde and H_2 by a H_2-producing bacterium, called S organism, is obligatorily linked to CH_4 formation by the H_2-utilizing methane-forming bacterium, i.e., no alternative electron sink exists for the monoculture of the ethanol-oxidizing S organism. Interspecies H_2 transfer is obligatory for good growth of the ethanol oxidizer. S organism can use pyruvate as an energy source in the absence of a methanogenic bacterium. The major products of pyruvate fermentation are ethanol, acetate, and CO_2 (11). In the presence of a H_2-utilizing, methanogenic bacterium, ethanol production by S organism is almost completely eliminated, and acetate production increases in an amount equivalent to ethanol produced by S organism alone (11). In the case of *R. albus*, interspecies electron transfer is not obligatory for growth on glucose, but it does cause a similar shift from ethanol to acetate formation and probably also in the amount of energy (ATP) available to the carbohydrate-fermenting organism for biosynthetic purposes.

It remains to be proven, however, that *R. albus* does contain an NAD-linked hydrogenase activity. Preliminary experiments (unpublished data) show that NAD or nicotinamide adenine dinucleotide phosphate (oxidized form) can be reduced by H_2 with cell-free extracts of *R. albus*, but only when methyl or benzyl viologen is present. We will continue our efforts to demonstrate a physiological system for the reduction of pyridine nucleotides by hydrogen. We feel, however, that the results in this paper, together with the results of Reddy et al. (11–13) on the association of S organism with H_2-utilizing, methanogenic bacteria, provide strong evi-

dence for a type of interspecies electron transfer mediated by H_2 gas which can be of great importance in the determination of product formation in anaerobic ecosystems.

There appears to be little significant influence of growth rate on the kinds and amounts of products of glucose fermentation in the single or mixed cultures. Significant amounts of formate were not detected at any growth rate. Other investigators reported that formate was a major product of cellobiose fermentation in batch cultures of *R. albus* (1, 4, 9). Dehority, however, reported that *R. albus* strain 7 produced formate from cellobiose when cultivated in an atmosphere containing 100% CO_2, but no formate was produced when growth was limited by the presence of only 5% CO_2 in the gas atmosphere (4). Dehority suggested that the CO_2 concentration regulated the production of formate. This would not be the case in our continuous-culture experiments which were carried out in the presence of 100% CO_2. Preliminary experiments carried out by T. L. Miller in our laboratory show that *R. albus* strain 7 produces formate from glucose only during the late log and stationary phases of growth in batch culture in a medium identical to that used in the continuous-culture experiments except for the concentration of glucose. The amount of formate produced in batch culture depends on the amount of glucose added, i.e., how much residual glucose remains to be fermented in the late log and stationary phases. It is highly probable that steady-state growth on limiting glucose is the explanation for the lack of formate production by the continuous cultures of *R. albus*.

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