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# Glucose and Carbon Dioxide Metabolism by *Succinivibrio dextrinosolvens*

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**Growth rates and culture conditions affect the molar yields of catabolic end products and cells of *Succinivibrio dextrinosolvens* growing on glucose. When growth in chemostats occurred, a trend toward decreased succinate and acetate formation, increased lactate formation, and a higher yield of cells correlated with an increase in the growth rate. End product and cellular yields on defined medium indicate a high maintenance requirement for *S. dextrinosolvens* and are consistent with energy conservation steps during the formation of acetate and succinate. Simultaneous carbon dioxide consumption and production were determined from batch studies with $NaH^{14}CO_3$, and the amounts were used to calculate a fermentation balance. These data also indicated that $CO_2$ consumption lags behind $CO_2$ production early in the growth phase, becoming equivalent to it toward stationary phase. Significantly more $CO_2$ was fixed by *S. dextrinosolvens* when the organism was cultured in chemostats sparged with $CO_2$. Formate is in part derived from free $CO_2$ in the medium, as shown by $^{13}C$ nuclear magnetic resonance studies, and may be sensitive to $CO_2$ availability. Nuclear magnetic resonance data are consistent with the carboxylation of a $C_3$ intermediate of the Embden-Meyerhof-Parnas pathway of glycolysis to a $C_4$ compound to eventually form succinate.**

*Succinivibrio dextrinosolvens* is a gram-negative anaerobe isolated from the bovine rumen (3). It is often the predominant isolate from the rumen when the diet of the animal is high in starch (1, 2). It is not clear why a high-starch diet should favor the proliferation of *S. dextrinosolvens*, since large numbers of other starch- and dextrin-fermenting microorganisms already exist in the highly competitive rumen environment. Its success could be related to a number of factors, including maintenance energy requirements (18), acid resistance (21), substrate preferences and affinities (16, 17, 19), carbon flow regulation (12), and energy-yielding mechanisms (7, 13).

The end products of the overall rumen fermentation are the result of the metabolic activity of the rumen inhabitants and are important to the health and productivity of the animal. The importance of the activity of *S. dextrinosolvens* in the rumen can be inferred from its large numbers under certain circumstances and from knowledge of the roles of its end products (acetate, succinate, formate, and lactate). Acetate is readily absorbed from the rumen and subsequently used in fatty acid metabolism. Succinate and formate, on the other hand, are considered intermediates of rumen fermentation which are further metabolized by other organisms. Succinate is thought to be an important intermediate for propionate formation (20, 24), with the latter being readily absorbed from the rumen for gluconeogenesis. Formate has been shown to be metabolized to methane by rumen methanogens (8, 10). Lactate is also an intermediate of the overall rumen fermentation and, when present in excess, has been correlated with acidosis (21).

Previous qualitative studies of glucose fermentation by *S. dextrinosolvens* in pure culture show succinate and acetate as the major end products and formate and lactate as minor ones (3, 23). Scardovi identified end products and several enzyme activities from an unconfirmed strain of *S. dextrinosolvens* but was unable to obtain a fermentation balance (23). Our studies demonstrate the effect of the growth rate of *S. dextrinosolvens* on its end products and, by the measurement of $CO_2$ metabolism, facilitate obtaining a feasible fermentation balance. Furthermore, $^{13}C$ nuclear magnetic resonance (NMR) experiments present evidence for the glucose fermentation pathway used by *S. dextrinosolvens* and identify the sites in the pathway at which $CO_2$ is fixed.

## MATERIALS AND METHODS

**Chemicals and gases.** All chemicals were reagent grade and were purchased from Sigma Chemical Co., St. Louis, Mo., and Mallinckrodt, Inc., Paris, Ky. Substrates with stable $^{13}C$ isotopes (99.1 to 99.9% enriched) were purchased from Isotec, Inc., Miamisburg, Ohio. $NaH^{14}CO_3$ was purchased from New England Nuclear, Boston, Mass. Gases were purchased from Badger Welding, Madison, Wis.

**Organism and cultivation.** *S. dextrinosolvens* (ATCC 19716; purchased from the American Type Culture Collection, Rockville, Md.) was grown under anaerobic conditions at 38°C in either 300-ml chemostats or 165-ml anaerobic bottles. The growth medium contained the following (per liter): $KH_2PO_4$, 18 g; $(NH)_2SO_4$, 0.75 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.22 g; $CoCl_2 \cdot 6H_2O$, 0.008 g; $FeSO_4 \cdot 7H_2O$, 0.0046 g; trace mineral solution (9), 10 ml; $NaHCO_3$, 4 g; DL-methionine, 0.2 g; L-leucine, 0.4 g; L-serine, 0.3 g; 1,4-naphthoquinone, 0.007 g; vitamin solution (described below), 0.56 ml; cysteine hydrochloride, 1 g; glucose, 1.4 to 2.7 g; and resazurin, 0.02 g. All ingredients except bicarbonate, glucose, and cysteine were added, and the medium pH was adjusted to 7.0. The bicarbonate then was added, and the medium was dispensed either into bottles under a stream of 95% $N_2$ and 5% $CO_2$ gas and sealed or into 20-liter carboys (open) and autoclaved. Cysteine and glucose were autoclaved separately and added just prior to inoculation. In studies with $^{13}C$-labeled glucose or carbonate, the glucose or bicarbonate was omitted from the basal medium and the

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labeled compounds were filter sterilized and added to sealed bottles after autoclaving. The vitamin solution contained the following (per 100 ml of water): 0.036 g each of thiamine hydrochloride, calcium pantothenate, nicotinamide, riboflavin, and pyridoxal phosphate; 0.09 g each of biotin, folic acid, and thioctic acid; 0.0036 g of cyanobalamin; and 0.009 g of *p*-aminobenzoic acid. The inclusion of the three amino acids and of 1,4-naphthoquinone and *p*-aminobenzoic acid obviated the need to add clarified rumen fluid to the medium as a source of growth factors (5). For batch growth of *S. dextrinosolvens*, anaerobic bottles were pressurized to 10 lb/in$^2$ with a gas mixture consisting of 95% $N_2$ and 5% $CO_2$, except in tracer studies with $Na_2{}^{13}CO_3$, in which 100% $N_2$ was used as the headspace gas. Inocula for all batch experiments were obtained from steady-state glucose-limited chemostats.

Growth in chemostats was initiated by adding 50 ml of exponential-phase culture to a chemostat containing approximately 50 ml of fresh medium. A medium flow rate of 0.5 ml/min was maintained until the culture reached the operating volume of 300 ml. The chemostat was operated at the indicated dilution rate with a sparge of oxygen-free $CO_2$ gas at a rate of approximately 40 ml/min. Cell growth was monitored by optical density at 660 nm in 1-cm cuvettes and was correlated to dry weight (optical density of 1 = 0.73 mg [dry weight]/ml). Culture purity was routinely checked by microscopic analysis and end product profiling with high-performance liquid chromatography (HPLC; see below) and was confirmed periodically by observing isolates from anaerobic plating on the medium described above supplemented with 2% agar.

**Acid and glucose analysis.** Fermentation end products were identified and quantified by HPLC (60-μl injections) with a UV detector operating at 210 nm. A Bio-Rad HPX87H column at 44°C was used with 28 mM $H_2SO_4$ as the eluant at a flow rate of 0.6 ml/min. Glucose was measured with a hexokinase assay kit (Sigma Chemical Co.).

**$^{14}CO_2$ studies.** Sterile $NaH^{14}CO_3$ (2 μCi) was added to 50 ml of medium in sealed anaerobic bottles, and the mixtures were equilibrated overnight at 38°C. The bottles then were inoculated with 8 ml of *S. dextrinosolvens* from glucose-limited chemostats and allowed to equilibrate for 40 min before timing was begun and the first sample was taken. Throughout the incubation, 0.8-ml-gas and 1.2-ml-liquid samples were taken. Upon withdrawal of 0.8 ml of headspace gas, 0.4 ml was analyzed for $CO_2$ content on an HP5890 gas chromatograph with helium as the carrier gas and a 100/120 Carbosieve S II column (2.5 m by 0.3 cm; Supelco Inc., Bellefonte, Pa.) under conditions described previously (14), except that the detector was set at 220°C. The remaining 0.4 ml of gas was injected into scintillation vials which contained 0.2 ml of methanol-ethanolamine (4:1) to trap the $^{14}CO_2$. Liquid samples (0.5 ml each) were filtered through a 0.2-μm-pore-size filter and analyzed by HPLC and the hexokinase assay described above. At the same time, 100 μl of liquid sample was added to a scintillation vial treated with a pellet of dry ice, acidified with 0.5 ml of 50% $H_2SO_4$, and left uncapped until the dry ice was consumed. All counts were performed on a Packard Tricarb scintillation counter (Packard Instrument Co., Rockville, Md.) with Optifluor as the scintillant. An external set of standards was used for obtaining a quench curve. The efficiency of counting was 89%.

**Incorporation of $^{13}C$ substrates into end products and cellular material.** $^1H$-decoupled $^{13}C$ NMR spectroscopy was performed on supernatants from cultures that were incubated for 12 to 18 h in the presence of 15 mM [1-$^{13}C$]glucose, [2-$^{13}C$]glucose, or 25 mM [$^{13}C$]$Na_2CO_3$. Supernatants were filtered through a 0.2-μm-pore-size filter and frozen at −20°C until needed for acquisition of spectra. For obtaining spectra, 0.45 ml of the sample and 0.05 ml of deuterium oxide were added to 5-mm-diameter tubes.

Proton-decoupled, one-dimensional $^{13}C$ spectra were obtained with a Bruker 500-MHz spectrometer operating at 125.759 MHz. $^1H$ decoupling was accomplished with the WALTZ-16 sequence (25). The sweep width was 22 kHz, and the acquisition time was 0.164 s. A pulse angle of 45° and a 2.5-s delay were used. Data were zero filled to 64,000 before being Fourier transformed and were apodized with an exponential function with a line broadening of −10 Hz. The number of scans varied from 125 to 6,500, according to the concentration of label expected. The chemical shifts and peak height ratios of the samples were compared with those from spectra of external standards acquired under identical conditions. Standards were suspended in fresh culture medium for catabolic products or in water at pH 1 for anabolic products. The chemical shifts were referenced relative to tetramethylsilane by using an external standard of [2-$^{13}C$]acetate at 23.659 ppm for catabolic products and, for anabolic products, an internal standard of natural-abundance D-glucose with the $C_1$ (β-form) resonance set at 96.05 ppm.

Cells were fractionated according to the procedure described by Roberts et al. (15) for determining label position in glutamate and aspartate. The protein residue was acid hydrolyzed, and aspartate and glutamate were separated from nonacidic amino acids with a Dowex AG1-X8 acetate column according to the procedure described by Weimer and Zeikus (29). Fractions containing glutamate and aspartate were pooled, rotary evaporated to dryness, and suspended in 0.5 ml of water containing 100 mM glucose, acidified with 1 drop of 1 M HCl, and added to 5-mm-diameter tubes with deuterium oxide as described above for obtaining spectra.

## RESULTS

**Fermentation products.** In order to observe the effects of the growth rate of *S. dextrinosolvens* on the distribution of carbon in its fermentation end products, samples were taken from steady-state continuous cultures at various growth rates (equal to dilution rates under steady-state conditions; Fig. 1). The same reservoir of medium containing approximately 8 mM glucose was used for all of the growth rates shown. Although the concentrations of total carbon recovered in end products were similar for all of the growth rates (52 mM at $\mu = 0.428$ and $\mu = 0.308$ h$^{-1}$ and 48 mM at $\mu = 0.164$ and $\mu = 0.117$ h$^{-1}$), a trend in end product distribution correlated with an increase in the growth rate. For example, a shift in the amount of carbon from succinate and acetate to cell material occurred with an increase in the growth rate. In addition to accumulating more cell material, faster-growing cells produced more lactate. The amount of formate produced in these continuous cultures was two to four times greater than that of earlier reports for batch cultures. Other products were not observed (even when a refractive index detector was applied), except for trace amounts of malate and fumarate.

A quantitative comparison of the fermentation products of *S. dextrinosolvens* grown as described above at specific growth rates of 0.1 and 0.46 h$^{-1}$ is shown in Table 1. The two growth rates approximate in vivo rates that *S. dextrinosolvens* may achieve in the rumen, 0.1 h$^{-1}$ (doubling time, 6.9 h) being slightly above the dilution rate of liquid contents in

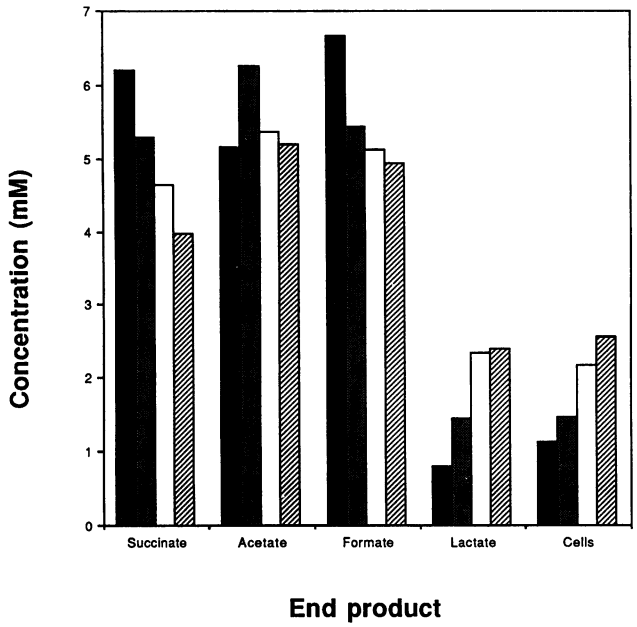


FIG. 1. Effect of growth rate on measured fermentation products of *S. dextrinosolvens* in glucose-limited continuous culture. The conditions are described in footnote *a* of Table 1. ■, $\mu = 0.117\ h^{-1}$; ▨, $\mu = 0.164\ h^{-1}$; □, $\mu = 0.308\ h^{-1}$; ▨, $\mu = 0.428\ h^{-1}$.

the rumen and $0.46\ h^{-1}$ (doubling time, 1.5 h) being near the maximum growth rate for *S. dextrinosolvens* (under the conditions tested in this laboratory), which it may approach in the rumen during transient bursts of nutrient abundance. Compared with the lower growth rate, the cell yield at the higher growth rate was two times greater and the molar ratio of lactate formed to glucose used was more than six times greater, whereas the succinate production was 50% less.

Relative to the amount of glucose used, the fermentation products listed in Table 1 have more carbon and are more oxidized; in agreement with previous reports (3, 23), this indicates that *S. dextrinosolvens* uses carbon dioxide as a substrate. This assumption and the data given in Table 1 were used in applying three different methods to estimate the

TABLE 1. Quantitative comparison at two growth rates of the end products of glucose fermentation by *S. dextrinosolvens*[a]

| Product | Amt (mmol) of product/100 mmol of glucose at a growth rate ($h^{-1}$) of: | |
|---|---|---|
| | 0.10 | 0.46 |
| Succinate | 113 | 57 |
| Acetate | 85 | 49 |
| Formate | 75 | 70 |
| Lactate | 3 | 23 |
| Cells[b] | 16 | 29 |

[a] Cells were grown in 300-ml chemostats at glucose concentrations (millimolar) of 9.13 ($\mu = 0.10\ h^{-1}$) and 10.3 ($\mu = 0.46\ h^{-1}$). The pH was 6.8 ± 0.10 throughout the test. Measurements were taken at steady state ($\mu = D$) as determined by constant optical density after several vessel turnovers. Glucose limitation was confirmed in separate experiments by observing an increase in growth upon glucose addition.

[b] Calculations for cells were based on dry weights calculated from $A_{660}$ readings and with the following general formula for cells: $C_5H_7O_2N$ (11). The percent cell yields (grams of cells per 100 g of glucose) were 10 and 18, respectively, at growth rates (per hour) of 0.10 and 0.46.

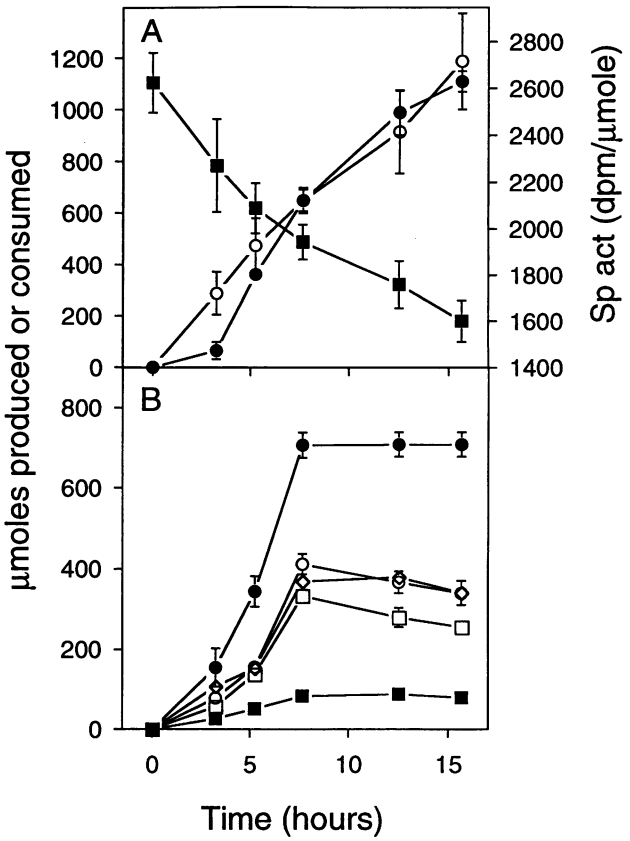


FIG. 2. Carbon dioxide metabolism (A) and fermentation end product analysis (B) of *S. dextrinosolvens* growing on glucose in batch culture. (A) ■, specific activity (sp act); ●, $CO_2$ consumed; ○, $CO_2$ produced; (B) ●, glucose; ○, succinate; ◇, acetate; □, formate; ■, lactate.

amounts of $CO_2$ fixed by *S. dextrinosolvens* in these continuous cultures. The validity of these estimates then was checked by using them in calculations for carbon recovery and, by using the convention O = 1 and H = −0.5, for oxidation/reduction ratios (redox ratios) of substrate to product. Included in these ratios is the value of −1.5 for cells, based on the general formula for cells, $C_5H_7O_2N$. For one of these methods, it was assumed that $[CO_2]_{fixed}$ = [succinate] + [formate] − [acetate], with the result that the carbon recovery and the redox ratio, respectively, at each growth rate were as follows: $\mu = 0.1\ h^{-1}$, 112% and 1.2; $\mu = 0.46\ h^{-1}$, 90% and 1.9. In another of these methods, the amount of $CO_2$ fixed was estimated as the amount required to balance the carbon recovery which resulted in redox ratios of 2.3 at $\mu = 0.1\ h^{-1}$ and 0.18 at $\mu = 0.46\ h^{-1}$. The fixation of $CO_2$ was estimated by a third method by setting it equal to the amount required to obtain a redox ratio of 1 which gave the most reasonable balance with carbon recoveries of 115% at $\mu = 0.1\ h^{-1}$ and 95% at $\mu = 0.46\ h^{-1}$.

**Fermentation balance.** The difficulties in obtaining a fermentation balance by using estimates of the amounts of $CO_2$ fixed prompted $^{14}CO_2$ tracer studies so that the metabolism of $CO_2$ could be monitored and quantitated. The results of a time course of $CO_2$ metabolism and acid production by *S. dextrinosolvens* growing on glucose experiment measuring are shown in Fig. 2A and B. The quantities of $CO_2$ production and fixation, which appear to vary independently

TABLE 2. Fermentation balance of *S. dextrinosolvens* at the end of exponential growth phase in batch culture on glucose[a]

| Substrate or product | Amt (μmol) of product/100 μmol of glucose |
|---|---|
| $CO_2$ (consumed)[b] | 92 |
| Succinate | 58 |
| Acetate | 52 |
| Formate | 47 |
| Lactate | 12 |
| $CO_2$ (produced)[c] | 92 |
| Cells[d] | 34 |

[a] All values are averages from $^{14}C$-labeling experiments ($n$ = 4).

[b] Cumulative $CO_2$ consumption at 7.67 h was calculated by the equation {[acid-stable dpm $ml^{-1}$ ($t_{7.67}$) − acid-stable dpm $ml^{-1}$ ($t_0$)]/specific activity of $^{14}CO_2$ ($t_{7.67}$)} × total liquid volume, where dpm is disintegrations per minute. The specific activity at each time point for 0.4-ml headspace gas samples was obtained by the disintegrations per minute per micromole of $CO_2$ that were determined.

[c] Cumulative $CO_2$ production at 7.67 h was calculated by the equation [total dpm added ($t_0$)/specific activity of $^{14}CO_2$ ($t_{7.67}$)] − [total dpm added ($t_0$)/specific activity of $^{14}CO_2$ ($t_0$)].

[d] Results for cells were calculated from a separate experiment under identical conditions, in which glucose consumption was correlated to cell mass determination ($n$, 4; standard error, 3.2 for molar yield and 2.0 for percent yield). The percentage of carbon recovered was 98%; the redox ratio of substrates to products was 0.8 (see text); the percent cell yield was 21 g of cells per 100 g of glucose.

throughout the course of the fermentation (Fig. 2A), correlate with the average balances (number of experiments, four) calculated at various time points. For example, a 92% carbon recovery and 0.8 redox ratio were calculated from the data at time ($t$) = 5.25 h when production exceeded fixation by 1.3-fold. By the time that the cells had exhausted the glucose supply and had begun to lyse ($t$ = 7.67 h), the $CO_2$ fixation-to-$CO_2$ production ratio was 1:1, and a 100% carbon recovery and 0.8 redox ratio (Table 2) were calculated.

The specific growth rate for the period from 0 to 7.67 h in these batch experiments (0.37 $h^{-1}$) was close to that of the fast-growing chemostat culture given in Table 1 (0.46 $h^{-1}$). The molar yields of succinate and acetate between the two modes of growth are similar, whereas that of formate in batch culture is much less than that in chemostat culture (although near that reported earlier in batch experiments [23]).

**Incorporation of $^{13}C$ substrates into catabolic and anabolic end products.** One-dimensional $^{13}C$ NMR spectroscopy was performed on the supernatants of batch cultures after their growth on isotopically labeled substrates (Fig. 3A and B) to elucidate the fermentation pathway of *S. dextrinosolvens*. When C-1-labeled glucose was the substrate, label was found primarily in the C-2,3 of succinate, the C-3 of lactate, and the C-2 of acetate (Fig. 3A; Table 3). When glucose labeled in the C-2 position was the substrate, the C-2,3 of succinate again contained the majority of label, whereas lactate and acetate were labeled primarily in their C-2 positions (Fig. 3B).

When the labeled substrate was $Na_2CO_3$, the majority of label appeared in the C-1,4 of succinate. From these later experiments, it was possible to obtain the fraction of the total acid which is labeled by dividing the end product quantity indicated by NMR data referenced to external standards by the total amount of acid indicated by the HPLC data. The data from one batch culture thus analyzed indicated that 36% of the total formate formed was labeled (data not shown). Approximately 15% of the total lactate was labeled in the C-1 position in these experiments.

Because of nuclear-overhauser enhancement, equivalently labeled carbon atoms will produce different signal intensities based on the number of protons bonded to them; thus, peak heights representing these intensities will vary according to the order $CH_3 > CH_2 > CH > C$. The signal intensity of a carboxyl carbon is lessened even further by the need for long relaxation times. Therefore, to estimate the distribution of label in products enriched with label in more than one position, peak height ratios (from height measurements in millimeters) from spectra of test samples were compared with those of natural-abundance standards acquired under identical conditions. The ratios were normalized to the carbon atom of the standard with the shortest peak height and were used to calculate the percent $^{13}C$ incorporated into each of the carbon atoms of the products listed in Table 3. Values for percent label incorporation from growth on 2-$^{13}C$-labeled glucose (data not shown) were 78 and 22% for the C-1 and C-2 of acetate and the C-2 and C-3 of lactate and 100% for the C-2,3 of succinate.

The results are consistent with the formation of two $C_3$ units from glucose via the Embden-Meyerhof-Parnas pathway and the carboxylation of a $C_3$ unit to a $C_4$ intermediate forming succinate. However, the occurrence of minor amounts of label in the C-2 of lactate from [1-$^{13}C$]glucose and in the C-2 of acetate and the C-3 of lactate from [2-$^{13}C$]glucose are not consistent with the operation of only the Embden-Meyerhof-Parnas pathway. A source of these minor amounts of label might be a $C_4$ intermediate whose label is randomized through fumarate and is subsequently decarboxylated, forming a $C_3$ compound that can be converted to acetate.

One way to test this hypothesis is to examine the distribution of label in anabolic products whose precursors may be from the same pool of labeled 4-$C_4$ intermediates as those that are decarboxylated to form $C_3$ compounds. The resultant distribution of label in aspartic and glutamic acids from cells grown on C-1-labeled glucose is shown in Fig. 4 and summarized in Table 3. If the pathway to their formation proceeds through oxaloacetate, then the C-3 and C-2 of aspartate share the same origin, respectively, as the C-2 and C-3 of glutamate. The degree of randomization in these analogous positions is the same for glutamate and aspartate and is close to that seen in the catabolic products (Table 3). These results support the hypothesis that the source of the minor peaks in the $C_2$ and $C_3$ compounds may be equilibrium reactions with label-randomized $C_4$ intermediates. In agreement with these results are those from $Na_2{}^{13}CO_3$-labeled cells which showed that the C-1 and C-4 of aspartic acid were equivalently labeled and that glutamic acid was labeled solely in the C-1 position.

## DISCUSSION

Obtaining a glucose fermentation balance for *S. dextrinosolvens* has been facilitated by quantitation of its simultaneous fixation and production of $CO_2$ in batch cultures. Although it was complicated by the significant effect of the growth rate on end product ratios, it was possible from the data in batch cultures to derive a representative mass balance for the net fermentation 1 glucose + 0.92 $CO_2$ → 0.92 $CO_2$ + 0.58 succinate + 0.52 acetate + 0.47 formate + 0.12 lactate + 0.34 cells. Although the carbon recovery derived from this balance was on target, the redox ratio of products to substrates was low (Table 2). This might be due

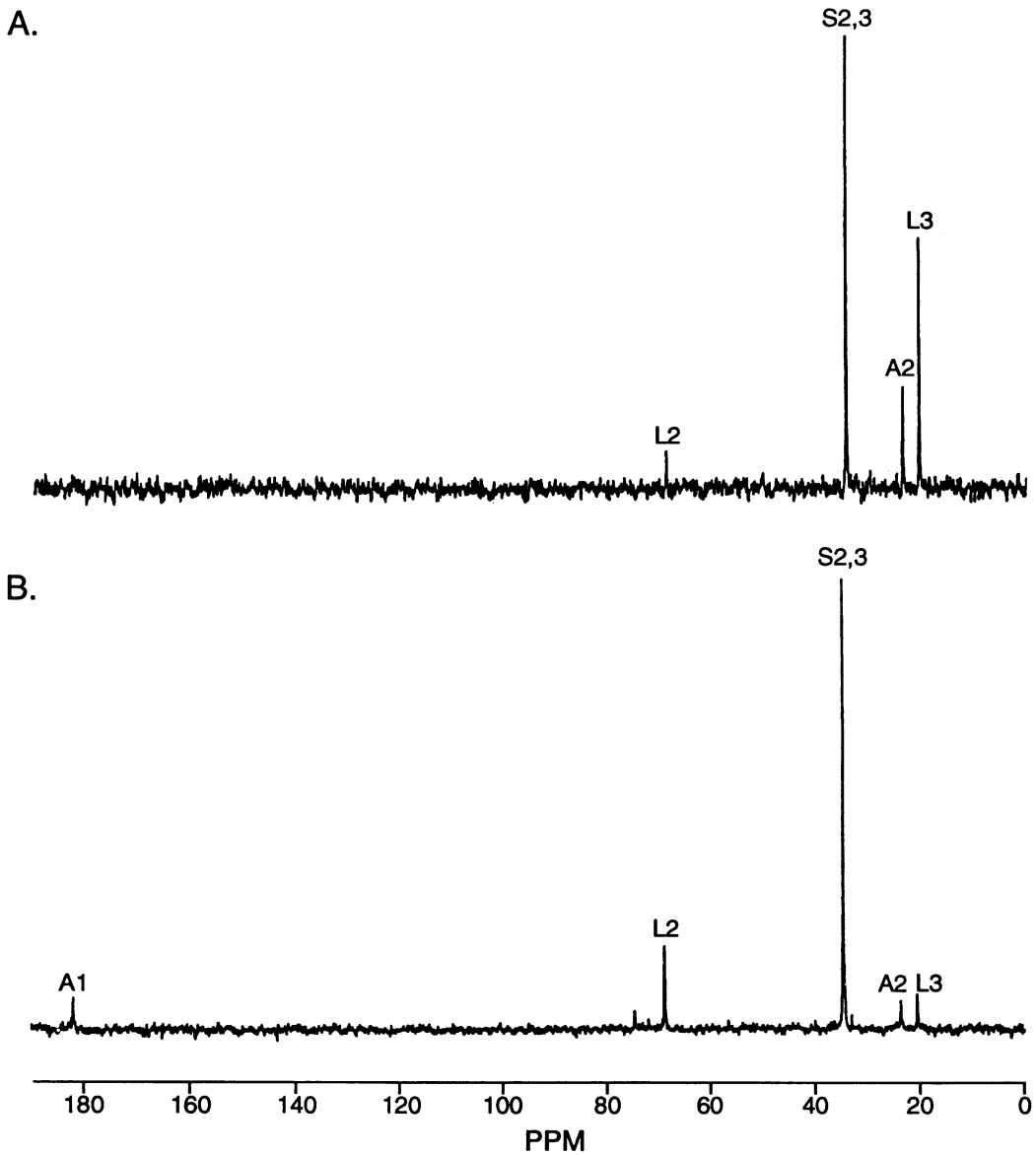


FIG. 3. Positions of $^{13}C$ in end products from catabolism of [1-$^{13}C$]glucose (A) and [2-$^{13}C$]glucose (B). The C-2 and C-3 of succinate cannot be distinguished because of the symmetry of the molecule. Spectra are representative of several experiments. A1, C-1 of acetate; A2, C-2 of acetate; L2, C-2 of lactate; L3, C-3 of lactate; S2,3, C-2,3 of succinate.

to an underestimation of the redox values of the substrates because of the way in which $CO_2$ fixation was calculated. These calculations made use of the specific activity of $CO_2$ in the headspace which may be slightly greater in value at a given time than the specific activity of that in the liquid because of a lag in the equilibration of $CO_2$ between the liquid and gas phases. Consistent with this explanation is that the addition of the value representing the small amount of $CO_2$ (10 μmol) necessary to obtain a substrate-to-product redox ratio of 1 to the value representing the calculated amount of $CO_2$ fixed shown in Table 2 still results in a reasonable carbon recovery of 97%.

Balancing redox levels with values for net $CO_2$ consumption was the method which likewise resulted in the best overall balances in the chemostat studies (Table 1). The resultant estimates of the molar consumption of $CO_2$ for the two growth rates shown in Table 1 were 82 μmol of $CO_2$ per 100 μmol of glucose for $\mu = 0.1\ h^{-1}$ and 42 μmol of $CO_2$ per 100 μmol of glucose for $\mu = 0.46\ h^{-1}$. Poor balances resulted if the molar yields of $CO_2$ fixation and production obtained in the batch studies (Table 2) were used to calculate balances for the chemostat studies (Table 1); redox ratios were near 0.5 and carbon recoveries ranged from 103 to 122%. Carbon dioxide metabolism between the two modes of growth may be different because of the large difference in the availability of $CO_2$ between the two culturing conditions (methods).

Maintenance coefficients may reflect the ability of an organism to survive in a competitive environment. Organisms with high maintenance requirements may be expected to be outcompeted by those with lower requirements when levels of soluble substrates are low (18). Maintenance coefficients and growth yield values were calculated with end

TABLE 3. Distribution of $^{13}C$ label within the metabolites of *S. dextrinosolvens* after growth on [1-$^{13}C$]glucose

| Metabolite analyzed[a] | % label in metabolite carbons[b] | | | | |
|---|---|---|---|---|---|
| | C-1 | C-2 | C-3 | C-4 | C-5 |
| Acetate | 0 | 100 | | | |
| Lactate | 0 | 16 | 84 | | |
| Succinate | 0 | 50 | 50 | 0 | |
| Aspartic | 0 | 30 | 70 | 0 | |
| Glutamic | 0 | 42 | 17 | 41 | 0 |

[a] Aspartic and glutamic percentage values are from the averages of two experiments, with standard errors of ± 3.0% or less. The values for the other metabolites are from the averages of three or more experiments, with standard errors of 2.5 for acetate and 0 for lactate.

[b] Signal intensity ratios were normalized to those obtained from natural-abundance standards under identical conditions. Natural-abundance peak intensity ratios were as follows: acetate, C-1 to C-2, 1:3.1; lactate, C-1 to C-2 to C-3, 1:1.96:2.39; succinate, C-1 and C-4 to C-2 and C-3, 1:3.32; aspartic acid, C-1 to C-2 to C-3 to C-4, 1:2.68:3.16:1.13; glutamic acid, C-1 to C-2 to C-3 to C-4 to C-5, 1.06:2.69:3.06:3.08:1.

product yield data from Fig. 1 and Table 1 and the assumptions that the molar yields of ATP per mole of succinate, acetate, and lactate are, respectively, 2, 2, and 1. The values obtained from these calculations are 14 g of cells per mol of ATP for $Y_{ATP}$, 0.78 g of glucose per g of bacteria per h for the maintenance coefficient, and a theoretical maximum yield of 51% with a correlation coefficient of 0.99. This maintenance coefficient is higher than that determined for several other rumen anaerobes (18). However, the medium composition has been shown to affect the value of maintenance coefficients (26), and thus the restrictive nature of the medium used in our studies may significantly raise the value above that expected for growth in the rumen or in the rich medium used in the previous studies. Nonetheless, the nature of the change of end product and cell yields as the growth rate increases is internally consistent with the high maintenance requirement. As seen in Fig. 1 and Table 1, cell yields increase as the growth rate increases, despite the shift in end products away from those whose formation is expected to include an energy conservation step (succinate and acetate). This type of shift was also seen with several other rumen anaerobes (*Selenomonas ruminantium* [12] and *Streptococcus bovis* [18]) and was cited as one of the difficulties in determining their maintenance requirements. A high maintenance requirement for *S. dextrinosolvens* may contribute to its being outcompeted when the level of soluble substrates in the rumen is low. Future studies based on these growth and maintenance data will be directed toward understanding the regulatory mechanisms at the branch points at which enzymes for the formation of lactate, acetate, and a $C_4$ compound may be competing for the same substrates.

Differences in formate yields at the different growth rates along with data from tracer studies provide evidence about the source of formate. First, since the formate and acetate yields vary independently (Fig. 1; Table 1), the reduction of $CO_2$ to formate may be uncoupled from the decarboxylation of pyruvate. In agreement with this hypothesis is the decrease in specific activity of $CO_2$ during the course of the fermentation (Fig. 2A), which indicates a release of unlabeled $CO_2$ and its subsequent equilibration with the bicarbonate-$CO_2$ in the medium. These data are consistent either with an oxidation of formate whose sole source is from a pyruvate formate lyase activity or with a release of $CO_2$ from the decarboxylation of pyruvate and the subsequent reduction of free $CO_2$ from the medium to make formate. Further evidence of the latter alternative comes from the $^{13}CO_2$ NMR experiments, which showed that 15% of the lactate formed is labeled in the C-1 position whereas 36% of the formate formed is labeled. Since the amount of lactate labeled in the C-1 position in these experiments should reflect the fraction of label resulting from exchange reactions into pyruvate and then into formate, its value of approximately 15% establishes that some of the labeled formate is coming from the direct reduction of dissolved $^{13}CO_2$ in the medium.

The amount of formate formed by *S. dextrinosolvens* may be sensitive to the availability of $CO_2$, as is the case for *Ruminococcus albus* (4). This is indicated by a comparison of its yield values in continuous and batch cultures (Tables 1 and 2). Over the range of growth rates in continuous cultures listed in Table 1 and Fig. 1, in which an atmosphere of 100% $CO_2$ flowing at 40 ml/min was provided, the yields of formate are nearly identical whereas those of the other end products differ widely. However, in batch culture in which a nonflowing atmosphere of only 5% $CO_2$ gas was provided, the formate yields were much lower than those of comparable

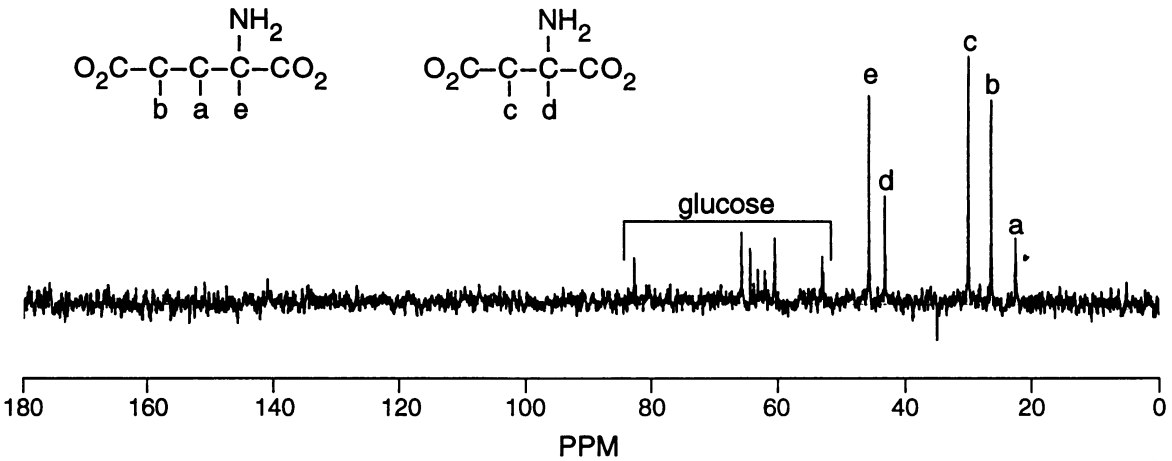


FIG. 4. Position of $^{13}C$ in the anabolic products aspartic acid and glutamic acid from the catabolism of [1-$^{13}C$]glucose. Natural-abundance-labeled glucose (100 mM) was added just prior to obtaining spectra to serve as an internal standard.

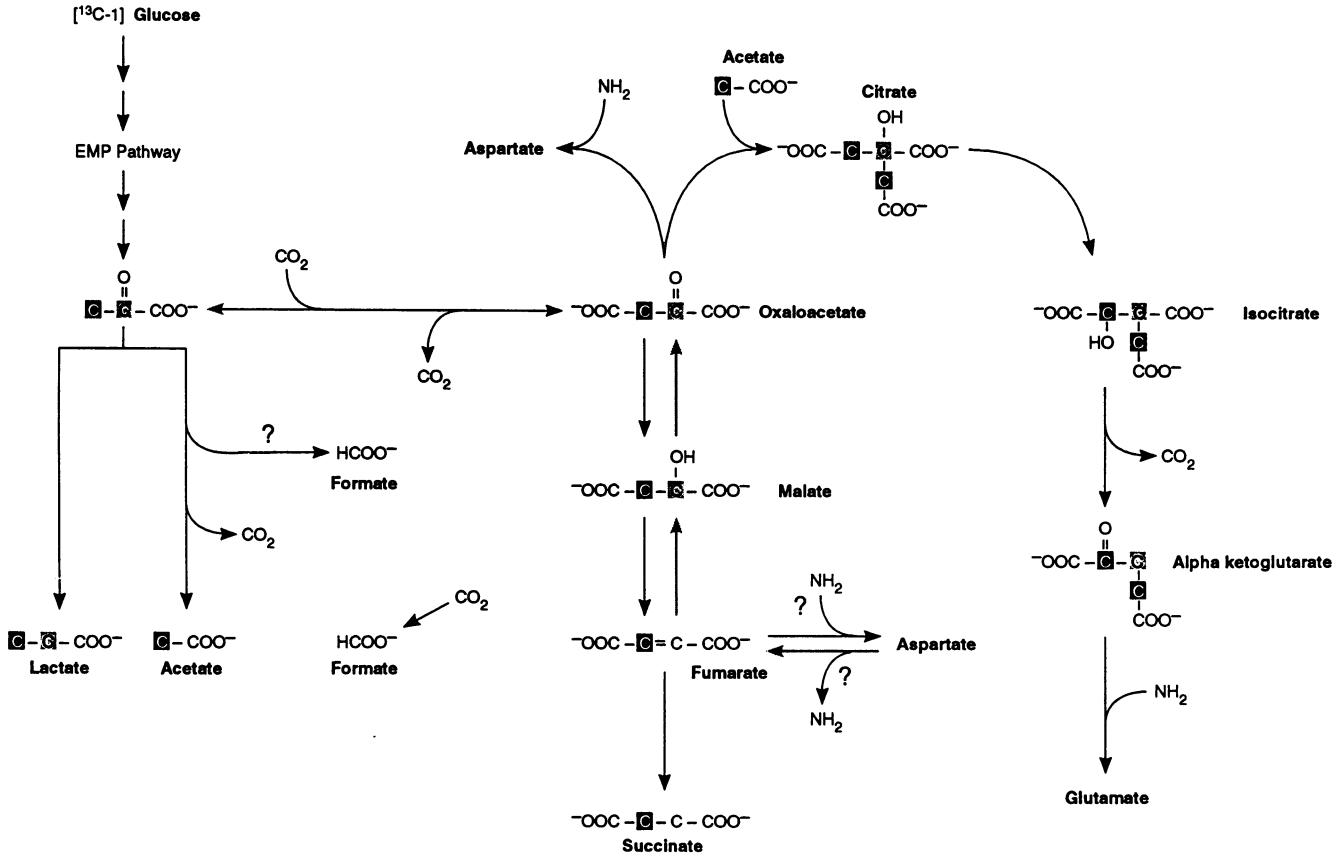


FIG. 5. Position of C-1 glucose in end products from Fig. 3 and 4 and summary of the pathway of glucose and $CO_2$ catabolism deduced from the data of all isotopic labeling experiments. ■, resultant position of the C-1 of glucose in products of metabolism; ▩, position of C-1 of glucose in products of metabolism after label is randomized; shaded arrow, possible pathways at which label randomization occurs. For clarity, the minor amount of label expected in the C-1 of acetate from randomization is not shown. EMP, Embden-Meyerhof-Parnas.

growth rate in continuous culture, even though those of succinate were not (Tables 1 and 2). Since bicarbonate-$CO_2$ is abundant in the rumen, the production of formate by *S. dextrinosolvens* may be a factor in the rate of methanogenesis in the rumen (8, 10) when starch levels are high. The differential effect of $CO_2$ availability on formate and succinate is the opposite of that recently reported for another succinate-producing anaerobe, for which succinate yields were much lower but formate yields were nearly unchanged with lowered $CO_2$ availability (22).

The NMR data suggest a pathway for the metabolism of glucose and $CO_2$ which is summarized in Fig. 5. These data are useful in directing subsequent enzymatic studies from which energy-yielding pathways can be deduced. The data in Fig. 3A and B show that the majority of label is in the C-2,3 carbon atoms of succinate whether the C-1 or C-2 of glucose is labeled. These results support the presence of the single carboxylation of a $C_3$ intermediate of the Embden-Meyerhof-Parnas pathway as a precursor to the formation of succinate, since in pathways in which a $C_2$ intermediate and two carboxylations are involved the C-2 of glucose would appear in the carboxyl carbons of succinate. Furthermore, *S. dextrinosolvens* incorporates $^{13}CO_2$ only into the carboxyl position of succinate (data not shown), which agrees with the reported enzymological data (23), whereas equivalent amounts of label would be seen in the C-2,3 as well as the C-1,4 carbon atoms if a $C_2$ precursor was being used. The labeling data in the present report do not rule out the operation of the acrylate pathway (28), except that complete randomization of label in aspartate would be expected. Other findings that support the model of the reductive tricarboxylic acid pathway to the formation of succinate over the acrylate pathway are the identification of a phosphoenolpyruvate carboxykinase in an unconfirmed strain of *S. dextrinosolvens* (23), the likely absence of a net yield of a high-energy bond in the acrylate pathway to succinate (27), and the failure under a variety of conditions to detect propionate, an expected intermediate of the acrylate pathway, although 0.01 to 0.1 mM amounts of fumarate and malate were detected. The labeling pattern in glutamate from $^{13}CO_2$ studies (data not shown) also provides evidence for the usual (S type) stereospecificity of citrate synthase, which is shown to be atypical (R type) in some anaerobes (6).

By illustrating the results of the [1-$^{13}$C]glucose data, Fig. 5 also shows how the observed pattern of labeling in the products detected could originate through label mixing at fumarate. The degree of randomization seen in the labeling patterns of aspartate and glutamate (Fig. 4; Table 3) support this hypothesis. However, an alternative source of the pattern of label observed in these amino acids could be from label mixing through a reaction involving the direct amination of fumarate by aspartase (Fig. 5). Further studies of

enzyme activities in extracts of *S. dextrinosolvens* are needed to reduce these options.

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