The Role of Carbonate in the Metabolism of Glucose by *Butyrivibrio fibrisolvens*

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The amount of Na_2CO_3 added to semi-synthetic medium determined the length of the lag phase, the growth rate and the dry weight of three strains of *Butyrivibrio fibrisolvens* (wv1, NOR37, B835). With increasing CO_3^{2-} concentration the molar growth yield of bacteria, from glucose, was increased and, of the fermentation products, formate increased more than the other acids. CO_3^{2-} -limited cultures of strain wv1 (Group 2 *Butyrivibrio*) and strain NOR37 (Group 1 *Butyrivibrio*) incorporated $^{14}\text{CO}_3^{2-}$ into lactate and formate. In NOR37, lactate and formate had equal specific activities; in wv1, the formate specific activity was twice that of lactate. Strain wv1 had an active pyruvate synthase and an energy-dependent exchange between CO_3^{2-} and formate was demonstrated. In strain wv1 butyrate was produced mainly from glucose.

INTRODUCTION

Many rumen bacteria require an exogenous source of CO_2 for growth (Dehority, 1971). *Butyrivibrio* was originally described as a group which did not require exogenous CO_2 (Bryant & Small, 1956) but subsequently some strains were shown to require exogenous CO_2 and others to be stimulated by it (Shane, Gouws & Kistner, 1969; Dehority, 1971). Exogenous CO_2 is used by rumen bacteria for the synthesis of cell material (Wright, 1960; Dehority, 1971) or may take part in reactions leading to metabolite formation (Caldwell, Keeney & Van Soest, 1969; Miller & Wolin, 1973; Howlett *et al.*, 1976).

Shane *et al.* (1969) subdivided the genus *Butyrivibrio* into two groups differentiated by their nutritional requirements and cellobiose fermentation products. Briefly, Group 1 strains have simple nutritional requirements, utilize acetate and produce, in addition to butyrate, large amounts of lactate and small amounts of formate. Group 2 strains are nutritionally fastidious and produce acetate and butyrate with small amounts of lactate and large amounts of formate.

The large numbers of butyrivibrios found in rumen contents of roughage-fed animals (Caldwell & Bryant, 1966; Van Gylswyk, 1970; Latham, Sutton & Sharpe, 1974) suggest that this genus contributes a significant proportion of the microbial protein available to the animal and participates in the production of butyrate and the interconversion of acetate and butyrate (Latham & Legakis, 1976; Van Gylswyk, 1976). Consequently the mechanisms

of energy and end-product generation in the butyrovibrios are significant features of rumen metabolism which deserve further study.

Asmundson (1974) observed that the molar growth yield of *Butyrovibrio fibrisolvens* wv1 (a Group 2 strain) strongly depended on the amount of CO_2 in the medium and that appreciable labelled carbon from $^{14}\text{CO}_3^{2-}$ appeared in the volatile fatty acid fraction of the growth medium. This paper describes the effect of carbonate on the fermentation of glucose by strain wv1 and two other strains of *B. fibrisolvens*.

METHODS

Media. The semi-synthetic medium 10 of Caldwell & Bryant (1966) was modified to the following composition [g (100 ml)^{-1}]: Trypticase (BBL), 0.2; yeast extract (Difco), 0.05; glucose, 0.1; cysteine, 0.025; resazurin, 0.00001; K_2HPO_4 , 0.237; KH_2PO_4 , 0.141; NaCl , 0.004; $(\text{NH}_4)_2\text{SO}_4$, 0.045; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.009; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.004; Na_2CO_3 , 0.16. Trypticase and yeast extract were dissolved in water, resazurin, KH_2PO_4 , $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ and the remaining minerals were added as separate solutions. This mixture was autoclaved at 121°C for 15 min and cooled to 40°C under O_2 -free CO_2 . Concentrated solutions of Na_2CO_3 , cysteine and glucose were sterilized by filtration and immediately added to the autoclaved, cooled medium, to produce the liquid medium A. This medium contained approximately 4 mM-acetate, 0.7 mM-propionate and 0.8 mM-lactate which were present in the Trypticase and yeast extract as contaminants.

Solid medium for roll tubes and slopes was prepared by adding 2% (w/v) agar to medium A before it was autoclaved.

For experiments on CO_3^{2-} metabolism, liquid medium A without Na_2CO_3 was prepared and handled under O_2 -free N_2 (medium B). The required levels of CO_3^{2-} were obtained by adding appropriate volumes of filter-sterilized Na_2CO_3 solution. Medium B minus glucose was used to wash bacteria free from carbonate.

Bacteria. Three strains of *Butyrovibrio fibrisolvens* were used: strain wv1 was isolated in New Zealand by Dr R. T. J. Clarke; strain NOR37 was isolated in Reading by Dr M. E. Sharpe; strain B835 was from the culture collection of the Rowett Research Institute. The purity of each strain was verified from colonial and morphological characteristics. Isolated colonies in roll tubes prepared from high dilutions of each culture were inoculated into liquid medium and incubated at 37°C for 24 to 48 h, transferred to agar slopes, incubated for a further 24 to 48 h and then frozen at -70°C . These frozen slopes served as stock cultures. To prepare an inoculum, a stock culture was thawed and subcultured twice in 10 ml of liquid medium under 100% CO_2 . The second subculture was washed twice and resuspended in 3 to 5 ml of anaerobic wash medium under N_2 . Inocula which became oxidized during preparation were discarded.

Cultural methods. The effect of CO_3^{2-} on growth and metabolism was studied in culture vessels of 150 to 160 ml capacity, consisting of a cylindrical body (approximately 35 mm diam.) with a short neck fused to one end and a cuvette fused to the other. These vessels were sterilized by autoclaving, cooled and flushed with N_2 for 3 to 5 min before sterile pre-reduced medium was added. Medium B (37 ml), containing glucose, was transferred to the culture vessel with a 10 ml syringe and the vessel was closed with a rubber stopper while a stream of N_2 was entering it. All subsequent additions were made by syringe through the stopper. The different levels of CO_3^{2-} required were obtained by adding 2.8 ml of sterile aqueous solution containing various proportions of filter-sterilized 2.12% (w/v) Na_2CO_3 solution.

Completed medium was incubated for 24 h at 39°C to check sterility. The vessels were then inoculated by injecting 0.1 ml washed bacterial suspension. Each vessel was immediately mixed by inversion, sampled for assay of initial glucose concentration and then incubated in a water bath at 39°C .

Analytical methods. Culture turbidity was measured *in situ* using an EEL colorimeter fitted with a dark blue filter (440 to 500 nm). Bacterial dry wt was determined when turbidity measurements indicated that growth had ceased. The culture (37 ml) was centrifuged at 20000 g for 20 min at 5°C , the supernatant was removed for analysis and the pellet was washed with 10 ml distilled water. The washings were discarded. The cells were transferred quantitatively to a weighed glass tube, recentrifuged at 9000 g for 50 min at room temperature and the pellet was dried overnight at 75°C then weighed.

Glucose was measured by the glucose oxidase method of Morley, Dawson & Marks (1968). Acetate, propionate and butyrate were assayed by gas-liquid chromatography (Fell *et al.*, 1968). The concentrations of volatile fatty acids were deduced from peak areas by comparison with standard curves. Formate was measured by the colorimetric method of Barker & Somers (1966), ethanol by the enzymic method of Kaplan & Ciotti (1957) and lactate by the microdiffusion method of Conway (1957).

Carbon dioxide was measured by the NaOH-trapping method of Dawes, McGill & Midgley (1971). Their apparatus was adapted to deal with samples from culture vessels by attaching a test-tube fitted with a side arm to the gas inlet of the bead tower. The test-tube was closed by a rubber stopper bearing a 13 gauge needle

which extended to the base of the tube. Samples (2.0 ml) for analysis were injected through the stopper with a hypodermic syringe and N_2 was bubbled through the needle into the sample at a rate of about 200 bubbles min^{-1} . The sample was acidified by injecting 1.0 ml 0.1 M-HCl and the CO_2 was transferred quantitatively, by bubbling for 20 min, to 10.0 ml 0.01 M-NaOH. CO_2 was estimated by back titration using phenolphthalein indicator in the presence of 2.0 ml 0.1 M- BaCl_2 . When radioactively labelled substrates were used, the suspension of BaCO_3 was made up to 50.0 ml and sampled for radioactivity measurements.

Preparation of silicic acid columns. The method used was based on that of Bové & Raveux (1957). Silicic acid (4 g, 100 mesh, AR; Mallinckrodt, St Louis, U.S.A.) was mixed with 0.25 M- H_2SO_4 (1 ml) and shaken in an Erlenmeyer flask to disperse the H_2SO_4 through the silicic acid. The acid-treated powder was mixed with 25 ml chloroform (washed with distilled water) and the suspension was poured into a glass column plugged at the lower end with lipid-free cotton wool. When the silicic acid had partially settled it was stabilized by eluting with a further 40 ml chloroform. The samples of mixed fermentation acids were loaded after the chloroform had drained to the level of the silicic acid in the column.

Analysis of radioactivity in fermentation end-products. Samples (2 ml) of bacteria-free culture media were made alkaline and evaporated to dryness. Each dry sample was acidified and transferred to a silicic acid column by Pasteur pipette. The sample tube was rinsed with chloroform and the chloroform was also added to the silicic acid column. The volatile fatty acids and lactic acid were separated by eluting the column sequentially with 25 ml chloroform containing 4, 8 or 15 % (v/v) *n*-butanol. Samples (2 ml) of eluate were collected in polypropylene scintillation vials and titrated with 0.005 M-methanolic NaOH, using phenol red as indicator. The neutralized samples were evaporated to dryness, and 0.1 ml water was added to redissolve the salts, followed by 10 ml scintillation fluid.

Radioactivity counting. All samples were counted in scintillation fluid [toluene, 1 litre; Triton X-100, 0.5 litre; 2,5-diphenyloxazole (PPO), 3.0 g; and 1,4-di-2-(5-phenyloxazolyl)benzene (POPOP), 0.3 g] on a Packard Tri-carb liquid scintillation counter, making appropriate corrections for quenching.

Aqueous samples (1.0 ml) were counted in 9.0 ml scintillation fluid. Column eluate samples were made up to 0.1 ml and counted in 10 ml scintillation fluid. Gas phase radioactivity was displaced by a slow stream of N_2 and trapped in 18 % (v/v) ethanolamine; samples (0.5 ml) of ethanolamine solution were diluted with an equal volume of water and made up to 10.0 ml with scintillation fluid. Radioactivity incorporated in bacteria was measured in triplicate samples (0.4 ml) of cell suspension which were collected and washed with distilled water (3×1 ml) on membrane filters (0.22 μm); each filter was dried briefly at 75 °C before being placed in 10 ml scintillation fluid. When these methods were used to determine the distribution of radioactivity between liquid, vapour and cellular phases in duplicate glucose-labelled cultures of *B. fibrisolvens*, the sums of the counts recovered from the cultures were 94.3 and 101.8 % of the counts added.

RESULTS

Medium

Preliminary experiments with *B. fibrisolvens* strain wv1 (Asmundson, 1974) in Caldwell & Bryant (1966) medium containing 5.55 mM-glucose showed a marked response to addition of Trypticase up to 0.2 % (w/v) and a much smaller response above this level. Thus 0.2 % Trypticase is sufficient to provide the organic nitrogen required in the medium.

In 0.2 % Trypticase medium, glucose up to 20 mM was growth-limiting. As our medium routinely contained 5.55 mM-glucose our results were obtained under conditions of energy-limited growth.

The buffering capacity of Caldwell & Bryant (1966) medium was inadequate when used to prepare CO_3^{2-} -limited cultures under N_2 due to the decreased contribution of the $\text{HCO}_3^-/\text{CO}_2$ buffer system and the low concentrations of PO_4^{3-} . When strain wv1 was grown in basal medium containing 5.55 mM-glucose and 2.78 or 5.55 mM- CO_3^{2-} , the final pH was 4.9. When the phosphate buffer concentration was increased to 24 mM with CO_3^{2-} concentrations of 0.1, 1.11, 2.78, 5.55, 22.2, 33.3 mM, the final pH values were 6.8, 6.7, 6.4, 6.5, 6.8, 6.8 respectively. Such changes in pH were considered unlikely to cause changes in glucose metabolism and the medium was used routinely with this buffer concentration.

Effect of CO_3^{2-} concentration on growth rate and bacterial yield

Each strain of *B. fibrisolvens* used in this study required exogenous CO_3^{2-} . The lag phase, growth rates and molar growth yields of the three cultures were each proportional to the

Table 1. *Effect of CO₃²⁻ concentration on growth rate and molar growth yield in B. fibrisolvens*

Strain	CO ₃ ²⁻ concn (mm)	Growth rate (h ⁻¹)	Yield [g (mol glucose) ⁻¹]
wv1	1	0.05	24.0
	2	0.19	35.0
	4	0.26	42.0
	10	0.44	48.0
	14	0.38	49.0
B835	1	0.06	ND
	2	0.10	28.5
	4	0.11	28.6
	10	0.09	29.0
	14	0.08	30.0
NOR37	1	0.08	30.0
	2	0.22	35.0
	4	0.44	36.0
	10	0.37	39.0
	14	0.47	42.0

ND, Not determined.

CO₃²⁻ concentration (see Table 1). The requirement for CO₃²⁻ by B835 was met at concentrations below those required by NOR37 and wv1.

Retention of carbon from glucose and CO₃²⁻

Cultures were grown in anaerobic culture vessels on basal medium containing glucose (5.55 mM) and CO₃²⁻ (4.0 mM) under an atmosphere of O₂-free N₂ with [U-¹⁴C]glucose or ¹⁴CO₃²⁻ being added before inoculation. Growth was terminated at the start of the stationary phase. When the distribution of ¹⁴C was determined in the medium, in the gas phase and in the bacteria (Table 2), relatively little ¹⁴C from ¹⁴CO₃²⁻ was found in the bacteria. With strain wv1, ¹⁴C from [U-¹⁴C]glucose was mainly in the bacteria-free liquid phase.

Thus under these conditions *B. fibrisolvens* (wv1) metabolized most of the glucose to produce energy and little of it was used to form new cell material. Similarly, although CO₃²⁻ was essential for growth, little of it was incorporated into cell carbon which suggested that CO₃²⁻ might have a role in energy production by these bacteria.

Effect of CO₃²⁻ limitation on glucose fermentation products

Duplicate cultures of each of the three strains of *B. fibrisolvens* were grown to the beginning of the stationary phase in the anaerobic culture vessels on the glucose-limited basal medium containing six different CO₃²⁻ levels ranging from 0 to 14 mM. The quantity of fermentation products formed with each organism (Fig. 1) was proportional to the glucose used and carbon balances indicated that an average of 89.6 % of the glucose consumed was accounted for in the products measured. Each strain produced butyrate as a major fermentation product. NOR37 produced a relatively high proportion of lactate and a low proportion of formate even at the highest CO₃²⁻ concentrations tested. Although it produced a little acetate, NOR37 conformed to Group 1 of Shane *et al.* (1969) since Latham & Legakis (1976) showed that under certain conditions Group 1 strains can produce small amounts of acetate instead of utilizing it. Strains wv1 and B835 produced acetate, a relatively low proportion of lactate and a high proportion of formate, so these two strains conformed to Group 2 of Shane *et al.* (1969). No cultures produced significant quantities of ethanol. Propionate did not appear to be metabolized or produced by these strains of *B. fibrisolvens*.

In each culture, the increase in CO₃²⁻ concentration was accompanied by an increase in the proportion of formate in the end-products. This was particularly noted in cultures of

Table 2. Distribution of carbon from $^{14}\text{CO}_3^{2-}$ or $[\text{U-}^{14}\text{C}]\text{glucose}$ in cultures of *B. fibrisolvens*

Radioactivities in the various culture components were determined as described in Methods.

Strain	Substrate	Specific activity (d.p.m. μmol^{-1})	Radioactivity recovered (% of total)			Carbon incorporation [$\mu\text{g (ml culture)}^{-1}$]
			Bacteria	Growth medium	Gas phase	
wv1	$^{14}\text{CO}_3^{2-}$	55875	3.9	49.0	46.7	2.1
	$[\text{U-}^{14}\text{C}]\text{glucose}$	45045	8.5	80.0	4.3	33.9
NOR37	$^{14}\text{CO}_3^{2-}$	31481	4.2	35.8	60.0	2.0

Fig. 1. Effect of CO_3^{2-} concentration on the glucose fermentation products of *B. fibrisolvens* strains: (a) wv1; (b) NOR37; (c) B835. Product concentrations ($\mu\text{mol ml}^{-1}$) have been corrected for levels present in basal media: $\circ$, acetate; $\bullet$, butyrate; $\square$, lactate; $\blacksquare$, ethanol; $\triangle$, formate.

B835 and wv1. In wv1 cultures at CO_3^{2-} concentrations above 2 mM, the formate concentration was higher than that expected from the stoichiometric production of acetate, butyrate and formate from pyruvate. Similar results were obtained for strains NOR37 and B835 with 14 mM- CO_3^{2-} .

*Incorporation of $^{14}\text{CO}_3^{2-}$ into fermentation end-products in *B. fibrisolvens* wv1 and NOR37*

After batch growth of *B. fibrisolvens* wv1 and NOR37 for 24 h at 39 °C in basal medium containing 4.0 mM- $^{14}\text{CO}_3^{2-}$ (20 μCi per 40 ml medium), the radioactivities and concentrations of the acid end-products in the media were assayed (Table 3). *Butyrivibrio fibrisolvens* wv1 produced acetate and little lactate, whilst strain NOR37 used acetate and produced high concentrations of lactate. The distribution of ^{14}C between formate and lactate

Table 3. *Incorporation of $^{14}\text{CO}_3^{2-}$ into end-products of fermentation of *B. fibrisolvens**

Strain		Fermentation end-products			
		Formate	Acetate	Lactate	Butyrate
wv1	Concn ($\mu\text{mol ml}^{-1}$)	3.75	0.64	0.29	3.40
	Specific activity* (d.p.m. μmol^{-1})	33 948	—	17 464	—
NOR37	Concn ($\mu\text{mol ml}^{-1}$)	4.33	—0.09	2.20	2.53
	Specific activity* (d.p.m. μmol^{-1})	4 829	—	5 166	—

* Specific activities corrected for the basal levels of formate and lactate in the medium.

differed in the two strains: lactate and formate had similar specific activities in strain NOR37 indicating that the pyruvate pool was the common precursor of both lactate and formate; with strain wv1, the lactate was labelled to a much lesser degree than the formate, indicating that formate may be labelled by additional pathways. Little or no labelling was noted in acetate and butyrate produced by either culture.

*Relationship between formate and CO_3^{2-} specific radioactivities in batch cultures of *B. fibrisolvens* wv1*

Batch cultures (120 ml) of *B. fibrisolvens* wv1 were grown under N_2 in CO_3^{2-} -limited conditions with $^{14}\text{CO}_3^{2-}$ ($0.166 \mu\text{Ci ml}^{-1}$) added at 4 or 10 mM either early in the exponential phase or early in the stationary phase. The concentrations and radioactivities of formate and CO_3^{2-} were followed. (As a control for measuring $^{14}\text{CO}_3^{2-}$ specific activities, a batch of sterile medium containing $^{14}\text{CO}_3^{2-}$ was also serially sampled.) Figure 2 shows the results of the experiment using 10 mM- CO_3^{2-} . The incorporation of ^{14}C into formate was related to the growth of the culture, but if the ^{14}C was added in stationary phase none was found in formate. When $^{14}\text{CO}_3^{2-}$ was added early in exponential phase to a culture containing 4 mM- CO_3^{2-} , formate became labelled to a maximum specific activity of 600 d.p.m. μmol^{-1} although the CO_3^{2-} specific activity was 16000 d.p.m. μmol^{-1} . The specific activity of the CO_3^{2-} decreased with time; part of this drop in specific activity may be due to the method used to sample the culture as there was a small decrease in specific activity in the sterile medium used as a control.

*Incorporation of [^{14}C]acetate into end-products of fermentation in *B. fibrisolvens* wv1*

To test for a functional pyruvate synthase in *B. fibrisolvens* wv1, 40 ml medium A containing 10 mM- CO_3^{2-} and $0.8 \mu\text{Ci}$ [^{14}C]acetate were inoculated and incubated at 39°C for 24 h. The radioactivities and concentrations of fermentation end-products were measured (Table 4). There was only a small net production of lactate in this culture but there was a significant incorporation of radioactivity indicating that acetate was converted to pyruvate which was then reduced to lactate. No activity was incorporated into formate and there was a low incorporation of ^{14}C into butyrate in this culture.

*Distribution of [^{14}C]glucose radioactivity among the end-products of fermentation in *B. fibrisolvens* wv1*

To test whether pyruvate or acetate added to the medium would diminish the requirement for CO_3^{2-} , batch cultures of *B. fibrisolvens* wv1 were grown to early stationary phase in 40 ml basal medium containing either 2 mM- CO_3^{2-} or 4 mM- CO_3^{2-} or 2 mM- CO_3^{2-} plus either 2 mM-sodium pyruvate or 2 mM-sodium acetate; [^{14}C]glucose was added at $0.97 \mu\text{Ci}$ per flask. The radioactivity in the acid end-products in the bacteria-free culture media (Table 5) varied between 58 and 77 % of the label in the added glucose. Added acetate

Fig. 2. Effect of growth phase on the specific activities of formate and CO_3^{2-} in cultures of *B. fibrisolvens* wv1. The CO_3^{2-} concentration in the medium was 10 mM and $^{14}\text{CO}_3^{2-}$ was added in early-exponential or early-stationary phase. Specific activities when $^{14}\text{CO}_3^{2-}$ was added to a growing culture: $\circ$, CO_3^{2-} ; $\bullet$, formate. Specific activities when $^{14}\text{CO}_3^{2-}$ was added to a culture in stationary phase: $\square$, CO_3^{2-} ; $\blacksquare$, formate. Specific activity when $^{14}\text{CO}_3^{2-}$ was added to an uninoculated culture vessel: $\triangle$, CO_3^{2-} . $\times$, $+$, Culture turbidity; arrows indicate the times at which $^{14}\text{CO}_3^{2-}$ was added.

Table 4. Incorporation of $[1-^{14}\text{C}]$ acetate into end-products of fermentation of *B. fibrisolvens* wv1

		Fermentation end-products			
		Formate	Acetate	Lactate	Butyrate
Concn ($\mu\text{mol ml}^{-1}$)	Total	7.50	4.80	0.85	2.88
	Net	5.60	0.65	0.07	2.88
Specific activity (d.p.m. μmol^{-1})		0	9397	2574*	176

* Specific activity corrected for the basal level of lactate in the medium.

Table 5. Distribution of $[U-^{14}\text{C}]$ glucose radioactivity among the end-products of fermentation in *B. fibrisolvens* wv1

Addition to basal medium	Growth rate (h^{-1})	Glucose used ($\mu\text{mol ml}^{-1}$)	Specific activity [d.p.m. ($\mu\text{mol C}^{-1}$)]			
			Formate	Acetate	Lactate	Butyrate
2 mM- CO_3^{2-}	0.20	4.22	1693	614	1065	1521
4 mM- CO_3^{2-}	0.30	5.76	1365	402	835	1685
2 mM- CO_3^{2-} + 2 mM-pyruvate	0.20	4.40	1595	464	1004	1359
2 mM- CO_3^{2-} + 2 mM-acetate	0.24	4.94	1147	346	519	1408

Specific activity of the substrate glucose was 1794 d.p.m. ($\mu\text{mol C}^{-1}$).

stimulated the growth rate and glucose utilization but less than did an equimolar addition of CO_3^{2-} . The specific activities of the end-products showed that butyrate was derived mainly from glucose and partly from a non-radioactive precursor, probably acetate present in the basal medium.

Formate and lactate specific activities were depressed by adding extra CO_3^{2-} or acetate. Adding pyruvate had no effect on lactate specific activity and caused only a small depression in formate specific activity.

DISCUSSION

Van Gylswyk (1976) has shown, using a Group 1 strain of *B. fibrisolvens*, that $^{14}\text{CO}_2$ can exchange with the pyruvate carboxyl carbon leading to production of labelled formate. Using a Group 2 strain (wv1) we found that uptake of $^{14}\text{CO}_3^{2-}$ into formate was growth dependent and, in the batches grown with 10 mM- CO_3^{2-} , the greater growth was accompanied by higher specific activities in formate although the specific activity of the added CO_3^{2-} was less than half that of the batch grown with 4 mM- CO_3^{2-} . The study of changes in CO_3^{2-} specific activity showed that as the formate specific activity increased so the CO_3^{2-} specific activity decreased. Examination of a control vessel in which samples of uninoculated media were assayed showed that part of this decrease was associated with the technique whereby medium removed was replaced with nitrogen. The decrease in CO_3^{2-} specific activity was, however, consistent with an exchange reaction.

Carbon dioxide may also enter pyruvate by way of pyruvate synthase and in strain wv1 [^{14}C]acetate was incorporated into lactate confirming that pyruvate synthase must be operating. Van Gylswyk (1976) found that pyruvate synthase operated in a Group 1 *B. fibrisolvens*.

If pyruvate is cleaved by pyruvate formate lyase then the formate and lactate produced from labelled pyruvate should be equally labelled. Group 1 *B. fibrisolvens* NOR37, grown in the presence of $^{14}\text{CO}_3^{2-}$, produced formate and lactate with equal specific activities indicating that pyruvate was a common precursor and confirming a similar result of Van Gylswyk (1976). In a similar experiment, however, using the Group 2 strain wv1, formate was more highly labelled than was lactate, indicating that formate was derived from $^{14}\text{CO}_3^{2-}$ by an additional path. Possibly in the Group 2 *B. fibrisolvens* CO_2 acts directly as an electron acceptor in a manner similar to that reported by Miller & Wolin (1973) for *Ruminococcus albus*. Miller (1975) reported that in *R. albus*, *R. flavefaciens* and *B. fibrisolvens*, formate did not arise directly by cleavage of pyruvate.

With strain wv1, grown in the presence of [^{14}C]glucose, additional pyruvate or acetate could not replace the requirement for CO_2 . Van Gylswyk (1976) showed that in a Group 1 *B. fibrisolvens*, external pyruvate could act as an electron acceptor and was rapidly converted to lactate. In strain wv1, external pyruvate did not enter the pool from which lactate was formed. It did, however, appear to be partly metabolized to acetate and thence to butyrate since the specific activities of these end-products were reduced. Added acetate reduced the specific activities of lactate and formate, the former presumably by means of the pyruvate synthase reaction, but it is not obvious how it would reduce the formate specific activity unless acetate carbon was converted to CO_2 by the operation of an acetate- CO_2 exchange (Ljungdahl & Wood, 1969) or by a tricarboxylic acid cycle pathway, reducing the specific activity of the CO_2 pool and thus the specific activity of formate. In each of these experiments there was evidence that butyrate was formed mainly from radioactive glucose and only partly from acetate present in the medium. Thus the Group 2 strain used in this study differed from the Group 1 strain used by Van Gylswyk (1976) which produced butyrate predominantly from added acetate rather than acetate derived from substrate glucose.

The increases in molar growth yields from glucose with strains wv1 and NOR37 with increasing CO_3^{2-} concentrations arose from concomitant increases in growth rate as, at

higher growth rates, less energy is diverted into maintenance functions (Pirt, 1965). With B835 there were only small changes in molar growth yield and growth rate with increasing CO_3^{2-} concentration as its CO_3^{2-} requirement was met at low levels; some other but unknown limitation was, however, affecting its growth.

Despite differences in the metabolic pathways which operate in Group 1 and Group 2 *B. fibrisolvens*, CO_3^{2-} can stimulate growth of both groups by acting as an electron acceptor. In the Group 1 bacteria, CO_3^{2-} is fixed by the reductive pyruvate synthase reaction producing pyruvate which can either be further reduced to lactate or split to acetate and formate. In the Group 2 bacteria, CO_3^{2-} is reduced directly to formate. In both groups of bacteria, H_2 can be produced but in pure cultures the hydrogenase reaction may become rate-limiting as H_2 accumulates, hence the requirement for formate production as an alternative means of regenerating oxidized co-factors.

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