JOURNAL OF BACTERIOLOGY, Oct. 1970, p. 50-56

Vol. 104, No. 1
Printed in U.S.A.

Biosynthesis of α-Ketoglutarate by the Reductive Carboxylation of Succinate in *Bacteroides ruminicola*

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Received for publication 13 April 1970

Experiments with growing cells and with cell-free extracts of *Bacteroides ruminicola* indicate that this anaerobic bacterium can synthesize α-ketoglutarate by a reductive carboxylation of succinate. When the organism was grown in medium containing succinate-*1,4*-^{14}C, most of the radioactivity in cells was in the protein fraction and most of the ^{14}C in protein was in the glutamic acid family of amino acids (glutamate, proline, and arginine). When unlabeled succinate was added to culture medium containing glucose-*U*-^{14}C, incorporation of radioactivity into the glutamic acid family of amino acids was greatly reduced. This supports the concept that succinate is an intermediate in synthesis of α-ketoglutarate. Cell-free extracts of the organism incubated with succinate-*1,4*-^{14}C incorporated ^{14}C into amino acids and most of this was found in glutamate. The cofactors which stimulate glutamate synthesis from succinate by extracts from these cells appear to be similar to the factors that have been demonstrated with extracts from photosynthetic bacteria. The position of label in glutamate synthesized from succinate-*1,4*-^{14}C, the probable absence of isocitric dehydrogenase, and studies with labeled citrate and with inhibitors of citric acid cycle enzymes support the concept of a reductive carboxylation of succinate as the only, or at least a major, mechanism for synthesis of α-ketoglutarate in this organism. This appears to be the first evidence for a net synthesis of α-ketoglutarate by this reaction in a nonphotosynthetic heterotrophic organism.

Bacteria belonging to the species *Bacteroides ruminicola* (8) are among the more numerous ruminal bacteria. Members of this species are obligate anaerobes that ferment a wide variety of carbohydrates and produce succinate, acetate and sometimes formate. *B. ruminicola* grows with ammonia as its main nitrogen source and is able to synthesize adequate quantities of all its amino acids except for methionine and possibly cysteine (29). The nicotinamide adenine dinucleotide phosphate (NADP)-linked glutamic dehydrogenase in this organism (23) is probably a major mechanism for fixation of ammonia. The apparent absence of isocitric dehydrogenase in one strain of this organism (23), however, raised a question concerning a mechanism for synthesis of α-ketoglutarate and thus glutamate and the glutamic acid family of amino acids. In this paper we present evidence that *B. ruminicola* is able to synthesize α-ketoglutarate by a reductive carboxylation of succinate. This reaction was first demonstrated in *Chlorobium thiosulfatophilum* (13) but has not been previously demonstrated in nonphotosynthetic organisms. A preliminary report of this work has been published (I. M. Robinson and M. J. Allison, Bacteriol. Proc., p. 128, 1969).

MATERIALS AND METHODS

Bacterial culture methods. *B. ruminicola* strains 23 and GA33 (8) were cultured at 38 C in the medium of Bryant and Robinson (7) as modified by Robinson and Allison (32). Strain 23 was the test organism except where specified otherwise. The anaerobic technique of Hungate (22) was used for cultures in test tubes. Large-batch cultures were grown in rubber-stoppered glass carboys (20 liters) or Erlenmeyer flasks (1 liter) equipped with cotton-plugged inlet and outlet tubes. The inlet tubes reached almost to the bottom of the flask. Media were sterilized in an autoclave and cooled while a slight pressure of CO_2 gas was applied to the head space above the liquid. Sodium carbonate was autoclaved separately as an 8% (w/v) solution and was cooled with slight N_2 gas pressure applied to the head space above the solution. This solution was gassed with CO_2 for 10 min or more before it was added to the rest of the medium. The cysteine-HCl·H_2O solution (1.25%, w/v) was also

sterilized separately under CO_2 and was maintained under CO_2 until it was added to the medium.

A 1-liter batch of medium was inoculated with a 20-ml culture and, after maximum growth was attained (usually 16 to 20 hr), was used as an inoculum for 19 liters of medium. Cells were harvested with a continuous-flow centrifuge system (Szent-Gyorgyi Blum; Ivan Sorvall, Inc.) usually 1 to 4 hr after cultures reached the maximum stationary phase of growth. Growth was estimated by measurement of optical density at 600 nm in rubber-stoppered test tubes (18 by 150 mm).

Radiochemicals. Succinic acid-*1,4-*^{14}C (4 mCi/mmole), L-glutamic acid-*U-*^{14}C (21 mCi/mmole), L-glutamic acid-*1-*^{14}C (4.8 mCi/mmole), and citric acid-*1,5-*^{14}C (30 mCi/mmole) were purchased from Calatomic (Los Angeles, Calif.). DL-Glutamic acid-*5-*^{14}C (4.7 mCi/mmole) was purchased from Volk Radiochemical Co. (Burbank, Calif.). The D-glucose-*U-*^{14}C (320 mCi/mmole) was purchased from Amersham/Searle Corp. (Des Plaines, Ill.).

Experiments with growing cultures. Cells from cultures grown in radioactive substrate (10 or 20 ml) were harvested by centrifugation, washed twice with anaerobic mineral dilution solution (6), and fractionated by the methods of Roberts et al. (31). Radioactivity was usually measured with a liquid scintillation spectrometer as previously described (32). Carbonate was precipitated as the barium salt, washed, dried, and weighed. The CO_2 released when acid was added to the $BaCO_3$ in a closed chamber (16) was trapped in ethanolamine-ethylene glycol monomethyl ether (2:1, v/v), and ^{14}C in this solution was measured using the liquid scintillation counter.

Chromatography. Amino acids were separated by paper chromatography by using the solvent systems of Wolfe (36) for two-dimensional chromatograms and the butanol-propionic acid-water system of Edwards et al. (17) for separations in one dimension. Radioactive areas on paper chromatograms were located by preparing radioautographs or by using a windowless, gas-flow, strip scanner. Radioactivity in areas located by means of a radioautograph was measured as follows. The area containing ^{14}C was cut from the chromatogram, placed at the bottom of a scintillation vial, and 0.5 ml of water was added to the vial. After 10 min, 15 ml of XDC scintillation solution (5) was added, and radioactivity was measured in the scintillation counter. This procedure is similar to that recommended by Cayen and Anastassiadis (15).

Diethylaminoethyl cellulose (DEAE)-treated extract was prepared as described by Buchanan et al. (12) except that anaerobic buffer was used and flowing N_2 was used to exclude air from the column and the collection tubes. Sephadex-treated extract was prepared by passing crude extract through a column of Sephadex G-25 (1.5 by 15 cm). The column was equilibrated with 0.02 M anaerobic phosphate buffer and was developed anaerobically as described above. Protein was measured by the method of Lowry et al. (27).

Experiments with cell-free extracts. Anaerobic phosphate buffer was a Na-K phosphate solution (0.02 M, *p*H 7.0, unless specified otherwise) which was heated to boiling, cooled, and then maintained under N_2 gas. Dithiothreitol (0.25 mg/ml) was added to this solution before it was used. Cells suspended in the anaerobic buffer (10 g of wet cell paste/20 ml) were exposed to a 20 kc sonic probe for 4 min under an atmosphere of N_2. The supernatant fluid after centrifugation at 25,000 × *g* for 10 min was used as the "cell-free extract."

Studies with cell-free extracts of *B. ruminicola* were conducted in test tubes (13 by 100 mm). The reactants were bubbled with N_2 for 45 to 60 sec, and then the cell extract was added and a rubber stopper was placed on the tube. Tubes were incubated at 38 C, usually for 60 min. The reaction was stopped with 0.3 ml of 12 N HCl. Tubes were placed at 4 C overnight and centrifuged at 10,000 × *g* for 15 min; the supernatant fluid was then analyzed. Radioactivity in amino acids was measured after separation of these from neutralized supernatant fluid by using Dowex 50 H^+ (31).

Glutamate degradation. Glutamic acid was isolated from protein hydrolysates and cell-free reactions by paper chromatography (36) and partially degraded. Carbon number 1 was removed by reaction with Chloramine-T by the method of Kemble and Macpherson (24). Carbon-5 was removed by reaction with a washed cell suspension of *Clostridium tetanomorphum* ATCC 15920 (34).

RESULTS

Experiments with growing cultures. The distribution of ^{14}C in fractions of *B. ruminicola* cells which were grown in medium containing succinate-*1,4-*^{14}C is given in Table 1. Carbonate in the supernatant fluid from the culture of strain 23 contained 2×10^6 disintegrations/min. The protein fractions, which contained most of the cellular ^{14}C, were hydrolyzed, and amino acids were separated by paper chromatography. Radioactivity was present in most amino acids in the protein hydrolysate, but the amino acids which belong to the glutamic acid family, glutamate, arginine, and proline, were more heavily labeled than any of the others (Table 2). Aspartate had about one-tenth as much ^{14}C as glutamate.

When unlabeled succinate (0.02 or 0.05 M) was added to cultures growing in medium containing ^{14}C-glucose, there was a marked decrease in the relative amounts of ^{14}C in glutamate, proline, and arginine. This was apparent by observation of radioautographs and was confirmed when the ^{14}C in radioactive areas from the chromatograms was measured (Table 3). The incorporation of radioactivity from glucose into amino acids not of the glutamic acid family was not appreciably reduced by adding unlabeled succinate to the growth medium.

Experiments with cell-free extracts. Cell-free extracts of *B. ruminicola* strain 23 incubated with

TABLE 1. *Incorporation of succinate-1,4-^{14}C by growing cultures of Bacteroides ruminicola and distribution of ^{14}C in fractions of the cells*[a]

Determination	Radioactivity (disintegrations/min)	
	Strain 23	Strain GA-33
Whole culture	16,872,000	6,960,000
Cell fractions		
Cold-trichloroacetic acid soluble	7,000	13,600
Alcohol-ether soluble	10,500	56,200
Hot-trichloroacetic acid soluble	12,700	32,000
Hot-trichloroacetic acid precipitate[b]	1,430,000	2,530,000

[a] The organisms were grown in 20-ml volumes and cells were harvested, washed, and fractionated (31) after incubation for 16 hr. The specific activity of succinate-*1,4-^{14}C* added to the medium at 0 hr was 4 mCi/mmole.

[b] Radioactivity in the hot-trichloroacetic acid precipitate (protein fraction) was measured after hydrolysis (20 hr) under N_2 at 105 C in sealed tubes.

TABLE 2. *Distribution of radioactivity in amino acids from Bacteroides ruminicola cells grown in medium containing succinate-1,4-^{14}C*

Amino acid[a]	Radioactivity[b]	Specific activity[c]
Glutamate	100.0	100.0
Arginine	34.4	84.4
Proline	30.8	87.0
Aspartate	11.4	11.1
Threonine	4.2	9.7
Isoleucine	3.1	5.4
Alanine	2.8	5.5
Valine	1.9	3.4
Lysine	1.3	1.6
Leucine	1.2	1.5
Phenylalanine	1.0	2.1
Tyrosine	0.8	1.8
Serine	0.8	2.6
Cysteic acid	0.7	4.7
Glycine	0.5	8.9

[a] The protein fraction (hot-trichloroacetic acid precipitate) from cells of *B. ruminicola* strain 23 (Table 1) was hydrolyzed and amino acids were separated by paper chromatography (36).

[b] Radioactivity relative to that in the glutamate area of the paper chromatogram (87,600 counts/min).

[c] Specific activity relative to specific activity of glutamate calculated from measurements of amino acid composition of *B. ruminicola* (30).

succinate-*1,4-^{14}C* incorporated ^{14}C into several amino acids, but most of this radioactivity was found in glutamate (Fig. 1). This finding was also confirmed by measurements of radioactivity in amino acids isolated after two-dimensional paper chromatography. The relative distribution of ^{14}C in glutamate, arginine, proline, and aspartate, respectively, was 83:7:4:6. The reaction was approximately proportional to the concentration of crude extract up to 3.2 mg of protein/ml, but inhibition of activity was found at 6.4 mg of protein/ml (Fig. 2).

Reductive carboxylation of succinate by

TABLE 3. *Effect of addition of unlabeled succinate to the growth medium on incorporation of isotope from ^{14}C-glucose into various amino acids*[a]

Chromatogram	Succinate added to culture medium	Ratio of radioactivity in given amino acid to radioactivity in alanine				
		Glutamic acid	Proline	Arginine	Valine	Aspartic acid
	M					
1	0	1.12	.42		1.19	1.11
2	0	1.25	.37	.40	1.13	1.22
3	0.02	0.32	.11		1.04	1.26
4	0.02	0.40	.093	.097	1.12	1.38
5	0.05	0.17	.054		1.12	1.37
6	0.05	0.19	.050	.057	1.20	1.39

[a] *B. ruminicola* strain 23 cells were grown on media containing glucose-*U-^{14}C*, and unlabeled succinate was added to two of the cultures as given. Amino acids in hydrolysates of protein from these cells were separated by paper chromatography (36), and ^{14}C in these was measured as described in the text. Values are expressed as the radioactivity of the ^{14}C in given amino acid divided by that of the ^{14}C in alanine from the same chromatogram. Measurements of ^{14}C in alanine ranged between 11,000 and 24,000 counts/min.

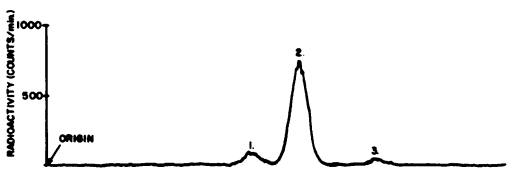

FIG. 1. *Scan of a paper chromatograph of labeled amino acids synthesized from succinate-1,4-^{14}C by a cell-free extract of B. ruminicola. Conditions were as given for the complete system in Table 4. Amino acids were separated from the reaction mixture by using Dowex 50 H^+, and the paper chromatogram was prepared by using the butanol-propionic acid-water solvent system (17). Peaks 1, 2, and 3 correspond to the locations of aspartate, glutamate, and proline, respectively.*

Sephadex-treated cell extracts was diminished when coenzyme A (CoA), adenosine triphosphate (ATP), triphosphopyridine, or the source of the amino group was omitted from the reaction mixture (Table 4). In another experiment, when glutamic dehydrogenase, EC 1.4.1.2 (100 μg, Calbiochem), was added to the reaction mixture, deletion of aspartate did not result in reduction of glutamate synthesis from succinate. With extracts which were not passed through a Sephadex column, these deletions had less effect except for deletion of pyruvate which greatly reduced the conversion of ^{14}C from succinate into amino acids by either crude or Sephadex-treated extracts. Labeled carbon from succinate was not incorporated into amino acids by crude or treated extracts unless air was excluded from the reaction vessel.

In one experiment, reactions were conducted in vessels that had wells containing NaOH to trap any CO_2 produced. The quantity of radioactivity from succinate-*1,4-^{14}C*, trapped as CO_2 (after acidification of the reaction mixture), was 7 and 7.4% of the amount of ^{14}C fixed into amino acids.

The results obtained from the partial degradation of glutamic acid synthesized by growing cells and glutamic acid synthesized by cell-free extracts incubated with succinate-*1,4-^{14}C* are given (Table 5). Carbon-1 of the glutamate contained little radioactivity. About one-half of the radioactivity in glutamate was in carbon-5 and the other half was in carbon-2, -3, or -4 which were not separated.

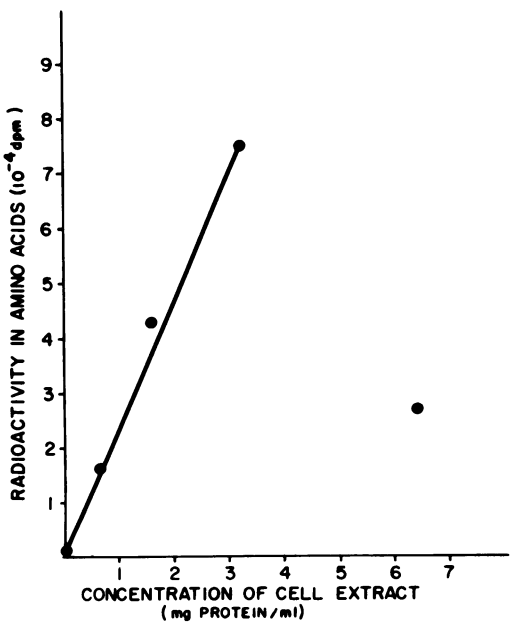

FIG. 2. *Succinate carboxylation as affected by the concentration of crude cell extract in reaction mixture. Conditions were as given in Table 6 with 50 μg of ferredoxin added to reaction vessels.*

TABLE 4. *Requirements for glutamate synthesis from succinate by extracts of Bacteroides ruminicola*

Reaction[a]	Radioactivity in amino acids (mainly glutamate)
	disintegrations/min
Crude cell extract (5.6 mg of protein)	
Complete	36,400
Sephadex-treated extract (2.8 mg of protein)	
Complete	24,700
Minus pyruvate	1,500
Minus TPP	13,000
Minus ATP	17,680
Minus CoA	7,800
Minus NH_3 and NADPH	11,440
Minus aspartate	10,900
Minus NH_3, NADPH, and aspartate	6,760
Boiled enzyme	1,300

[a] The complete reaction mixture contained (in μmoles): phosphate buffer, *p*H 7.0, 100; $MgSO_4$, 0.5; $MnCl_2$, 1; $CoCl_2$, 0.5; $NaHCO_3$, 10; sodium-pyruvate, 5; NH_4Cl, 10; reduced nicotinamide adenine dinucleotide (NADPH), 1; aspartate, 5; acetyl-coenzyme A (CoASH), 0.2; adenosine triphosphate (ATP), 0.5; triphosphopyridine (TPP), 1.0; and succinate-*1,4-^{14}C*, 5 (0.4 μCi), plus cell extract as indicated in a volume of 2.5 ml. Incubation was under N_2 for 60 min at 38 C.

Glutamate synthesis from succinate-*1,4-^{14}C* by crude cell-free extracts of *B. ruminicola* was usually stimulated when ferredoxin from *C. pasteurianum* was added to the reaction mixture (Table 6). When extracts were passed once through a DEAE cellulose column (1.2 by 5.5 cm), addition of ferredoxin produced a greater effect than with untreated, crude extracts but there was still not an absolute requirement for ferredoxin.

Assay of enzymes. Assays for isocitric dehydrogenase (EC 1.1.1.41 and EC 1.1.1.42) in extracts of *B. ruminicola* were attempted by using the spectrophotometric methods of Kornberg (26). Positive evidence for either the nicotinamide adenine dinucleotide (NAD)- or NADP-linked enzyme was not found. Several variations in the reaction mixture were tried including use of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and phosphate buffers,

TABLE 5. *Partial degradation of glutamic acid synthesized by Bacteroides ruminicola from succinate-1,4-^{14}C*

Source of glutamic acid	Degradation reaction[a]			
	Chloramine-T		*C. tetanomorphum*	
	CO_2= C1	C2 to C5	C1 to C4	CO_2= C5
Growing culture	2.8	97.2	52.0	48.0
Cell-free extract	4.6	95.4	53.4	46.6
Controls				
Glutamic acid-*1*-^{14}C	97.5	2.5	99.1	0.9
Glutamic acid-*5*-^{14}C	0.5	99.5	17.5	82.5
Glutamic acid-*U*-^{14}C	22.3	77.7	83.6	16.4

[a] Carbon-1 was recovered as CO_2 after reaction with Chloramine-T (24). Carbon-5 was recovered as CO_2 after reaction with a washed cell suspension of *Clostridium tetanomorphum* (34). The values given are the distribution (percentage) of ^{14}C recovered after each reaction.

TABLE 6. *Influence of ferredoxin on succinate carboxylation by extracts of Bacteroides ruminicola*

Reaction[a]	Radioactivity in amino acids (mainly glutamate)
	disintegrations/min
Expt 1	
Crude extract (8 mg of protein)	
No additions	50,000
Ferredoxin, 25 μg	58,200
Ferredoxin, 50 μg	75,100
Ferredoxin, 100 μg	50,000
Ferredoxin, 50 μg minus pyruvate.	6,400
Expt 2	
Crude extract (5.9 mg of protein)	
No additions	29,100
DEAE-treated extract (5.3 mg of protein)	
No additions	34,300
Ferredoxin, 1.5 μg	52,300
Ferredoxin, 15 μg	66,200
Ferredoxin, 45 μg	57,000
Methyl viologen, 1 μmole	28,000
Benzyl viologen, 1 μmole	2,600
Ferredoxin, 15 μg[b]	1,900
Ferredoxin, 15 μg[c]	500

[a] The reaction mixture and conditions were as given in Table 4 except that the volume in experiment 2 was 3.0 ml. The ferredoxin was from *Clostridium pasteurianum* (Worthington Biochemical Corp.).

[b] Incubated aerobically.

[c] Boiled cell extract.

deletion of Mg^{2+}, and addition of Mn^{2+} and adenosine monophosphate. An extract from a mixed population of ruminal bacteria obtained from a cow and purified isocitric acid dehydrogenase (Sigma Chemical Co.) were used as positive controls. Crude extracts from *B. ruminicola* did not appreciably inhibit the activity of the purified isocitric dehydrogenase. When a crude cell-free extract was incubated with citric acid-*1,5*-^{14}C (10 μCi), $NADP^+$, NAD^+, and aspartate, ^{14}C was not incorporated into amino acids.

No evidence for the presence of citrate synthase (EC 4.1.3.7) in *B. ruminicola* extracts could be found by using an assay system wherein fixation of ^{14}C-acetate was measured in the presence of oxaloacetate, CoA, ATP, glutathione, $MgCl_2$, and phosphate buffer (21).

No effect on synthesis of labeled glutamate from succinate-*1,4*-^{14}C was noted when either malonate (3.3 mM), fluorocitrate (2×10^{-2} to 16×10^{-2} mM), or Avidin (1.6 to 10 units/ml) was added to the succinate carboxylation reaction mixture.

DISCUSSION

Reductive carboxylations of acids to synthesize keto acids which are often precursors of amino acids have now been demonstrated in several laboratories. Mortlock and Wolfe (28) reported the net synthesis of pyruvate from acetyl phosphate and CO_2 by enzymes in extracts from *C. butyricum*. The strong reducing potential required for the carboxylation was supplied as the potent, but nonphysiological, reducing agent, sodium hydrosulfite. Bachofen, Buchanan, and Arnon (4) found that a natural low potential electron carrier, ferredoxin, could function in this "pyruvate synthase" reaction in *C. pasteurianum*. Since then, this reaction (equation 1) has been demonstrated in a number of other nonphotosynthetic and photosynthetic anaerobic bacteria (11).

$$\text{Acetyl-CoA} + CO_2 + \text{ferredoxin}_{\text{reduced}} \rightarrow \text{pyruvate} + \text{ferredoxin}_{\text{oxidized}} + \text{CoA} \quad (1)$$

$$\text{Succinyl-CoA} + CO_2 + \text{ferredoxin}_{\text{reduced}} \rightarrow \alpha\text{-ketoglutarate} + \text{ferredoxin}_{\text{oxidized}} + \text{CoA} \quad (2)$$

A similar ferredoxin-dependent reductive carboxylation of succinyl-CoA to synthesize α-ketoglutarate was found by Buchanan and Evans (13). This "α-ketoglutarate synthase reaction" (equation 2) is an important step in a recently described photosynthetic, carbon dioxide reduction cycle (18) and probably explains the labeling pattern in glutamate synthesized by *Rhodospirillum rubrum* from acetate and $NaHCO_3$ (20) or from succinate (33). Exchange reactions between

CO_2 and α-ketoglutarate have been demonstrated in extracts from *Escherichia coli* (1) and *Micrococcus lactilyticus* (35). It was suggested that this carboxylation reaction occurs in *Blastocladiella emersonii* (14), but there does not appear to be evidence for a net synthesis of α-ketoglutarate by this reaction in nonphotosynthetic organisms before our work.

Buchanan (10) recently described a similar ferredoxin-dependent reductive carboxylation of propionate to synthesize α-ketobutyrate. Reductive carboxylation reactions appear to be important for amino acid biosynthesis in several ruminal bacteria, and *B. ruminicola* was one of those that reductively carboxylated isobutyrate, isovalerate, and 2-methylbutyrate to synthesize valine, leucine, and isoleucine (2, 32). Other acids (acetate, phenylacetate, and indoleacetate) are carboxylated by ruminal bacteria to synthesize the carbon skeletons of alanine, phenylalanine, and tryptophan (2). Nonruminal photosynthetic bacteria, *Chromatium* and *R. rubrum*, also carboxylate phenylacetate to synthesize phenylalanine (3). Details of the mechanism of these reactions are not known, but with *Peptostreptococcus elsdenii* extracts, the carboxylation of isobutyrate is a ferredoxin-dependent reaction (Allison and Peel, *unpublished data*).

The concept that *B. ruminicola* synthesizes α-ketoglutarate by a reductive carboxylation of succinate is substantiated by results of experiments with growing cultures and with cell-free extracts. The organism possesses a glutamic-oxaloacetic transaminase (EC 2.6.1.1) and an NADP-linked glutamic dehydrogenase (EC 1.4.1.4) (23) and results (Table 4) suggest that both of these enzymes are functional in cell-free extracts. In experiments with labeled succinate, it is not likely that oxaloacetate was an intermediate between succinate and α-ketoglutarate because the specific activity of aspartate was much lower than the specific activity of the glutamic acid family of amino acids. The decreased incorporation of ^{14}C into the glutamic acid family of amino acids when unlabeled succinate was added to cultures growing in labeled glucose is strong evidence that succinate is an intermediate in biosynthesis of α-ketoglutarate. Since succinate is a major product of glucose catabolism by *B. ruminicola* (8), the exogenous pool of succinate would become labeled when the organism was grown in labeled glucose. Thus, even if succinate carboxylation were the sole mechanism for synthesis of α-ketoglutarate, one could not expect addition of unlabeled succinate to the medium to completely inhibit transfer of labeled carbon from glucose to α-ketoglutarate.

The position of ^{14}C in glutamic acid synthesized from succinate-*1,4-*^{14}C is further evidence for the reductive carboxylation reaction. About one-half of the radioactivity was in carbon-5 and the rest of the ^{14}C was in carbon-2 to -4, probably in carbon-2. This is the expected pattern from a reductive carboxylation sequence, but is different from the position of ^{14}C in glutamate that would be expected from forward reactions of the tricarboxylic acid cycle. If 1,4-labeled oxaloacetate was synthesized from succinate-*1,4-*^{14}C and this condensed with acetyl CoA to synthesize citrate with either the R or S condensing enzyme, citrate and isocitrate would be labeled in either carbon-1 or -6 and in carbon-4 (19). Half of the radioactivity (carbon-4) would then be lost as CO_2 by the isocitric dehydrogenase reaction and the resulting α-ketoglutarate would be labeled only in carbon-1 or -5, depending on the stereospecificity of the condensing enzyme. This was not the labeling pattern found, and, furthermore, there was little loss of ^{14}C as CO_2 while succinate-*1,4-*^{14}C was being converted to α-ketoglutarate.

We have not yet determined the nature of the low-potential electron carrier in *B. ruminicola*. A preliminary examination for ferredoxin in cells of this organism suggested that none was present (Allison and Peel, *unpublished data*). Extracts treated with DEAE cellulose retained some ability to carboxylate succinate without addition of ferredoxin, but the reaction was stimulated by ferredoxin from *C. pasteurianum*.

It will be necessary to purify the crude multienzyme system before reliable information on the mechanism of succinate carboxylation reaction can be obtained. We believe that in the cell-free system, pyruvate serves as a source of low-potential electrons for the reductive carboxylation of succinate, but it could also function as an energy source or as a carboxyl group donor. *B. ruminicola* extracts do not contain a hydrogenase, and hydrogen gas did not replace the requirement for pyruvate. Since extracts of this organism catalyze a rapid exchange reaction between pyruvate and CO_2, it is difficult to study succinate carboxylation by using $^{14}CO_2$ until a substitute for pyruvate is found.

The pattern of labeling in glutamate synthesized by *Methanobacterium omelianskii* [now known to be a symbiotic association of two organisms (9)] was not consistent with the operation of tricarboxylic acid cycle enzymes (25). A reductive carboxylation mechanism for synthesis of the isoleucine carbon skeleton has been demonstrated in *M. omelianskii* (32), and perhaps α-ketoglutarate is also synthesized by reductive carboxylation of succinate.

The position of labeling in glutamate synthesized by mixed ruminal organisms incubated with $NaH^{14}CO_3$ (L. P. Milligan, *personal communication*) supports the concept that the reductive car-

boxylation pathway proposed here functions and is quantitatively significant in the rumen. We have grown the following organisms in media containing succinate-*1,4-*^{14}C: *Selenomonas ruminantium* GA192 and HD4, *Methanobacter ruminantium* M1, *Ruminococcus flavefaciens* C94, *Bacteroides succinogenes* S85, *Butyrivibrio fibrisolvens* D1, *Streptococcus bovis* FD10, *Succinivibrio dextrinosolvens* 24, and *Borrelia* sp. B_25. Of these, only *S. ruminantium* incorporated labeled carbon into protein with more of the ^{14}C in the glutamic acid family than in aspartate or other amino acids. We thus suggest that *S. ruminantium* also reductively carboxylates succinate, but further work to substantiate this is needed.

ACKNOWLEDGMENTS

We are grateful to R. S. Hanson, University of Wisconsin, who confirmed our findings concerning isocitric dehydrogenase in *B. ruminicola* extracts. We also acknowledge the technical assistance of Jerry A. Bucklin.

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