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Fermentation of Fructose and Synthesis of Acetate from Carbon Dioxide by *Clostridium formicoaceticum*[1]

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Clostridium formicoaceticum ferments fructose labeled with ^{14}C in carbon 1, 4, 5, or 6 via the Embden Meyerhof pathway. In fermentations of fructose in the presence of $^{14}CO_2$, acetate is formed labeled equally in both carbons. Extracts convert the methyl groups of 5-methyltetrahydrofolate and methyl-B_{12} to the methyl group of acetate in the presence of pyruvate. Formate dehydrogenase, 10-formyltetrahydrofolate synthetase, 5,10-methenyltetrahydrofolate cyclohydrolase, 5,10-methylenetetrahydrofolate dehydrogenase, and 5,10-methylenetetrahydrofolate reductase are present in extracts of *C. formicoaceticum*. These enzymes are needed for the conversion of CO_2 to 5-methyltetrahydrofolate. It is proposed that acetate is totally synthesized from CO_2 via the reactions catalyzed by the enzymes listed above and that 5-methyltetrahydrofolate and a methylcorrinoid are intermediates in this synthesis.

Clostridium formicoaceticum ferments 1 mole of fructose, but not glucose, almost stoichiometrically to 3 moles of acetate (1, 5). Fructose-*1*-^{14}C and fructose-*6*-^{14}C are both converted to acetate labeled exclusively in the methyl carbon, and uniformly labeled fructose yields acetate labeled in both carbons (15). All of the enzymes of the Embden Meyerhof pathway were demonstrated in cell-free extracts of *C. formicoaceticum*, and it was concluded that the fermentation of fructose proceeds via this pathway (1, 15). Fermentation of fructose in the presence of $^{14}CO_2$ or ^{14}C-formate yields acetate labeled equally in both carbons (1, 15). These results indicate that *C. formicoaceticum* synthesizes acetate totally from CO_2. This hypothesis has recently been confirmed by using $^{13}CO_2$ and mass analysis, and about one third of the acetate is exclusively formed from CO_2 (26).

The above results are similar to those reported for *C. thermoaceticum*, which ferments glucose via the Embden Meyerhof pathway and synthesizes acetate from CO_2 according to the following reactions (2, 3, 32):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 8H + 2CO_2$$
$$2CO_2 + 8H \rightarrow CH_3COOH + 2H_2O$$
$$\text{Sum } C_6H_{12}O_6 \rightarrow 3CH_3COOH$$

The mechanism of the total synthesis of acetate from CO_2 has been extensively studied with *C. thermoaceticum*. These studies were reviewed recently (19). To form the methyl group of acetate, CO_2 is first reduced to formate. The formate is converted to 10-formyltetrahydrofolate, which is reduced to 5-methyltetrahydrofolate. The methyl group is then transferred to an enzyme-bound corrinoid, and finally carboxylation of the methyl group occurs to form acetate.

This report deals with the fermentation of fructose and the synthesis of acetate from CO_2 by *C. formicoaceticum*. Results will be presented of fermentations of fructose labeled with ^{14}C in carbon 1, 4, 5, or 6. Evidence will also be presented regarding the presence of enzymes necessary for the conversion of CO_2 to the 5-methyl group of 5-methyltetrahydrofolate and the formation of acetate from the latter as well as from methyl-B_{12}.

MATERIALS AND METHODS

Organism and growth conditions. *C. aceticum* isolated by El Ghazzawi (5) was used in these

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studies. This organism is now referred to as *C. formicoaceticum* (1). It was grown in a medium containing (in grams per liter): fructose, 12; $NaHCO_3$, 16; K_2HPO_4, 7.0; KH_2PO_4, 5.5; Difco yeast extract, 5; tryptone, 2.5; peptone, 2.5; $(NH_4)_2SO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; $Co(NO_3)_2 \cdot 6H_2O$, 0.028; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.007; sodium thioglycolate, 0.5; and boiled and filtered tomato juice, 50 ml. The medium was prepared in three solutions which were sterilized separately: A, containing fructose dissolved in 150 ml of water; B, containing sodium bicarbonate, the phosphates, and a few crystals of phenolphthalein in 250 ml; and C, containing all the other ingredients in 550 ml. Solution B was gassed aseptically with CO_2 just before use until the color of phenolphthalein was discharged. The sterile solutions were then combined and inoculated with 20 ml of a 48-hr culture. The cells were grown at 37 C for 72 hr with CO_2 bubbling through the medium. Harvest was by centrifugation in a Sharples continuous-flow centrifuge at 4 C. The unwashed cell paste from 20-liter cultures was stored at −20 C until it was used. The cell yield was about 2 to 4 g of wet cells per liter.

Fermentation with resting cells. Fermentations with intact (resting) cells were generally performed as follows. Three grams (wet weight) of freshly harvested cells was suspended in 40 ml of 0.1 M potassium phosphate, *p*H 7.0, containing 0.005 M cysteine. The suspension was placed in a 50-ml Erlenmeyer flask containing 2 mmoles of fructose. The flask was sealed with a serum stopper and gassed with argon and 2 mmoles of potassium bicarbonate in 1 ml of water was added with a syringe. The progress of the fermentation was followed by withdrawing samples at various times and by analyzing for fructose utilization by the anthrone method (12). Carbon dioxide from the fermentation was collected in a trap containing 3 ml of bicarbonate-free 3 N sodium hydroxide by bubbling air which was free of CO_2 through the acidified fermentation mixture. Acetic acid was obtained by steam distillation of the acidified fermentation mixture and was purified by chromatography on Celite (27). The specific radioactivities of fructose and of acetic acid samples were determined after oxidation to carbon dioxide (30). The specific activities of the individual carbons of acetate were obtained after degradation by the Schmidt procedure (23). The specific activity of CO_2 from the fermentations and from oxidations and degradations of acetic acid samples was determined by the procedure of Kornblatt et al. (13).

Enzymatic methods. Extracts of *C. formicoaceticum* were prepared by passing a suspension of 10 g of wet cells in 30 ml of 0.1 M potassium phosphate, *p*H 7.0, or 0.1 M potassium maleate, *p*H 7.0, at 25 C through a French pressure cell at 13,000 psi. The suspension of broken cells was centrifuged at 37,000 × *g* for 1 hr at 4 C. The dark-colored supernatant fluid was used for enzymatic assays without further fractionation. All assays were done at 37 C.

Formate dehydrogenase (EC 1.2.1.2) was assayed spectrophotometrically by measuring the increase of absorbancy at 340 nm with nicotinamide adenine dinucleotide (NAD) as the electron acceptor or at 600 nm with methyl viologen ($\epsilon = 11.3 \times 10^3$ M^{-1} cm^{-1}) (28). The reaction mixture contained (in micromoles in a volume of 1 ml): sodium formate, 20; dithiothreitol, 3; triethanolamine-hydrochloride (*p*H 7.5), 100; NAD, 1; or methyl viologen, 1.4. All solutions were made with water previously freed of oxygen by boiling and were stored under nitrogen. Anaerobic cuvettes were used and were sealed with serum stoppers and gassed with nitrogen before addition of enzyme with a syringe.

Formyltetrahydrofolate synthetase (EC 6.3.4.3) was assayed essentially as described by Rabinowitz and Pricer (25). The assay was performed in a volume of 0.5 ml containing (in micromoles): triethanolamine-hydrochloride (*p*H 8.0), 50; tetrahydrofolate, 0.5; sodium formate, 20; adenosine triphosphate (ATP), 2.5; magnesium chloride, 5; 2-mercaptoethanol, 50; and tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.4), 20. The last two compounds were added together with the tetrahydrofolate, which was stored in the Tris-hydrochloride buffer supplemented with mercaptoethanol. After 10 min of incubation, 1 ml of 0.36 N HCl was added to stop the reaction. The acidification converts the product of the reaction, 10-formyltetrahydrofolate, to 5, 10-methenyltetrahydrofolic acid, which was assayed at 350 nm ($\epsilon = 24.9 \times 10^3$ M^{-1} cm^{-1}).

Methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9) was measured by the decrease in absorbancy at 356 nm which occurs when 5,10-methenyltetrahydrofolate is converted to 10-formyltetrahydrofolate. The standard assay mixture contained in a total volume of 0.25 ml (in micromoles): potassium maleate, (*p*H 7.0), 50; 2-mercaptoethanol, 20; potassium hydroxide, 10; 5,10-methenyltetrahydrofolate, 0.05; added together with the 5,10-methenyltetrahydrofolate was 12 μmoles hydrochloric acid.

Methylenetetrahydrofolate dehydrogenase was assayed as described by Uyeda and Rabinowitz (29). The enzyme in *C. formicoaceticum* is specific for NAD, and there was no activity with nicotinamide adenine dinucleotide phosphate (NADP), which is the coenzyme for the enzyme isolated from *C. cylindrosporum* (29). The assay was performed in a total volume of 0.25 ml which contained (in micromoles): potassium maleate (*p*H 7.0), 50; NAD, 0.6; 5,10-methylenetetrahydrofolate, 0.24; 2-mercaptoethanol, 60. The reaction was followed by measuring the rate of formation of reduced NAD (NADH) and 5,10-methenyltetrahydrofolate, which both absorb at 356 nm. The combined molar extinction of these compounds at 356 nm is $\epsilon = 29.7 \times 10^3$ M^{-1} cm^{-1}.

Methylenetetrahydrofolate reductase was measured by following the conversion of 5-methyltetrahydrofolate labeled with ^{14}C in the methyl group to 5,10-methylenetetrahydrofolate (4, 11). The latter is in equilibrium with tetrahydrofolate and formaldehyde. The radioactive formaldehyde was trapped as the dimedon derivative. The enzyme reaction was performed in a small test tube in a total volume of 0.5 ml with the following components (in micromoles): menadione, 0.2; flavin adenine nucleotide (FAD), 0.25; sodium ascorbate, 2.5; potassium phosphate (*p*H 6.6), 50; -5-^{14}C methyltetrahydrofolate (27,400 counts per min per μmole), 0.5; and, added with the 5-methyltetrahydrofolate, 2-mercapto-

ethanol, 2.4. The reaction was allowed to proceed for 10 min, at which time 0.5 ml of a saturated dimedon solution in 0.1 M sodium acetate, *p*H 4.7, was added. The tubes were then immersed in boiling water for 3 min. After cooling, 3 ml of toluene was added to extract the radioactive dimedon derivative. The tubes were shaken vigorously and centrifuged. A 0.5-ml sample of the toluene layer was mixed with 10 ml of scintillation fluid and counted.

Formation of acetate from ^{14}C-methyltetrahydrofolate and ^{14}C-methylcobalamin was investigated exactly as described by Ghambeer et al. (7), except that the reaction was performed at 37 C instead of at 57 C as was the case with *C. thermoaceticum*.

Chemicals. Tetrahydrofolate was prepared by a modification of the procedure by Blair and Saunders (3). Six grams of folic acid was dissolved in 200 ml of 0.066 M Tris-hydrochloride, *p*H 7.8, and 6 g of sodium borohydride dissolved in 50 ml of water was slowly added while maintaining a slight pressure of nitrogen over the solution. After 20 min, acetic acid was added until a *p*H of 7.8 was obtained. 2-Mercaptoethanol was then added to a concentration of 0.5 M. The solution was passed through a diethylaminoethyl (DEAE) cellulose column (4.0 by 30 cm) previously equilibrated with 0.5 M 2-mercaptoethanol. The column was washed with 0.5 M 2-mercaptoethanol until the absorbancy at 298 nm of the eluate was nil. The tetrahydrofolate was then eluted with 0.2 M Tris-hydrochloride, *p*H 7.4, containing 0.5 M 2-mercaptoethanol. It was concentrated on a Diaflo ultrafiltration apparatus (Amicon Corp.) with a UM-05 filter. The concentration of tetrahydrofolate was assayed enzymatically with pure formyltetrahydrofolate synthetase isolated from *C. thermoaceticum* (17).

5,10-Methenyltetrahydrofolate was prepared enzymatically with formyltetrahydrofolate synthetase. The 10-formyltetrahydrofolate formed in the enzymatic reaction was converted to 5,10-methenyltetrahydrofolate by addition of hydrochloric acid. The acid solution was stored at −20 C and was used without further purification.

5,10-Methylenetetrahydrofolate was prepared by the method of Uyeda and Rabinowitz (29) by mixing 3 ml of 0.005 M tetrahydrofolate prepared as described above with 1 ml of 0.1 M formaldehyde. The mixture was left at 0 C for 1 hr before use.

Fructose-*1*-^{14}*C*, fructose-*6*-^{14}*C*, ^{14}C-methyl iodide, and ^{14}C-barium carbonate were from Mallinckrodt Chemical Works; ^{14}C-formaldehyde was from Amersham Searle. Fructose-*4*-^{14}*C* and fructose-*5*-^{14}*C* were obtained by exchange between fructose-6-phosphate and ^{14}C-labeled glyceraldehyde-3-phosphate, catalyzed by transaldolase (20). Methylcobalamin labeled in the Co-methyl group was synthesized from ^{14}C-methyl iodide and hydroxycobalamin (21). 5-Methyltetrahydrofolate labeled in the 5-methyl group was prepared as a free acid from ^{14}C-formaldehyde and folic acid (3). We also received a gift of methyltetrahydrofolate-*5*-^{14}*C* from D. Parker at Case Western Reserve University, which is gratefully acknowledged.

Dimedon, menadione, FAD, sodium pyruvate, ATP, NAD, folic acid, cobalamin, methyl viologen, and dithiothreitol were purchased from Sigma Chemical Co. All other chemicals were of analytical grade and were from different sources.

RESULTS

Whole cell experiments. The results summarized in Table 1 show that *C. formicoaceticum* ferments fructose almost stoichiometrically to acetate. Andreesen et al. (1) and Linke (15) have observed the formation of formate during fermentations of fructose by *C. formicoaceticum*. In our experiments, we did not observe any accumulation of formate, which is not surprising. Andreesen et al. (1) showed that the formation of formate depends on the state of the cells and that it occurs especially with cells in the stationary phase of growth and with washed resting cells. In accord with findings by Andreesen et al. (1) and Linke (15), we found that the fermentation of fructose was greatly stimulated by the presence of bicarbonate. Thus, about four times more fructose was fermented during the same time period in the presence of bicarbonate than was fermented in its absence. During the fermentation with bicarbonate, a high ratio of acetate formed to fructose fermented was observed. Presently, the only explanation for this observation is an endogenous formation of acetate from material present in the unwashed cells. This endogenous formation of acetate may be more pronounced when the concentration of bicarbonate is low. Other compounds may replace bicarbonate as electron acceptor and be converted to acetate.

When the fermentation of fructose occurs in the presence of ^{14}C-bicarbonate, the acetate produced is labeled in both carbons (Table 2). The distribution of ^{14}C between the two carbons of acetate is equal. These results are different from results obtained with *C. thermoaceticum*, which incorporates more $^{14}CO_2$ into the carboxyl than into the methyl group of acetate (18, 31).

Linek (15) demonstrated that *C. formicoaceticum* ferments fructose-*1*-^{14}*C* and fructose-*6*-^{14}*C* into acetate labeled only in the methyl group. Although these data are indicative of an Embden Meyerhof cleavage of fructose by the organism, they do not provide conclusive evidence. To further document that *C. formicoaceticum* employs and Embden Meyerhof pathway for the degradation of fructose, fructose-*4*-^{14}*C* and fructose-*5*-^{14}*C*, as well as fructose-*1*-^{14}*C* and fructose-*6*-^{14}*C*, were used as substrates for resting cells. The results are presented in Table 3. Each substrate was fermented in the absence and presence of a pool

of unlabeled CO_2.

When fructose-*1*-^{14}C and fructose-*6*-^{14}C were substrates, acetate was formed exclusively labeled in the methyl carbon as had been noted earlier by Linke (15). Fructose-*5*-^{14}C gave acetate labeled only in the carboxyl group. With fructose-*4*-^{14}C, a substantial amount of the label was trapped in the bicarbonate. The acetate formed during the fermentation of fructose-*4*-^{14}C had a low specific activity and was labeled in both carbons. The data presented in Tables 1, 2, and 3 are consistent with the proposal that fructose is fermented by *C. formicoaceticum* via the Embden Meyerhof pathway and that 2 moles of acetate are formed directly from carbons 1, 2, 5, and 6 of fructose and that a third mole of acetate is formed from CO_2.

Cell-free extract experiments. The outstanding characteristic of *C. thermoaceticum* is its capacity to convert the methyl groups of methyl-B_{12} (18, 24) and 5-methyltetrahydrofolate (7) to acetate. The data presented in Table 4 show that extracts of *C. formicoaceticum* also incorporate the methyl groups of 5-methyltetrahydrofolate and methyl-B_{12} into acetate. As observed with *C. thermoaceticum*, the formation of acetate from these substrates is dependent on the presence of pyruvate.

Enzymes involved in the conversion of CO_2 to the 5-methyl group of 5-methyltetrahydrofolate were assayed in extracts of *C. formicoaceticum*. As is seen in Table 5, all of the enzymes are present. The formate dehydrogenase and 5,10-methylenetetrahydrofolate dehydrogenase are specific for NAD as an electron acceptor, which could not be replaced by NADP. We have purified 10-formyltetrahydrofolate synthetase and 5,10-methylenetetrahydrofolate dehydrogenase almost to homogeneity (*unpublished data*), and these enzymes were tested under optimal conditions. The other enzymes have not been investigated further, and the activities listed must be considered as minimum.

TABLE 1. *Fermentation of fructose by cells of Clostridium formicoaceticum in the presence and absence of bicarbonate*[a]

Addition	Fructose fermented[b]	Acetate produced[b]		Moles product/mole substrate
		Actual	Theoretical	
+ HCO_3^-	0.412	1.104	1.236	2.77
+ HCO_3^-	0.376	1.042	1.128	2.77
− HCO_3^-	0.122	0.620	0.366	5.09
− HCO_3^-	0.100	0.570	0.300	5.70

[a] Fermentations were in 20 ml of 0.1 M potassium phosphate, *p*H 7.0, with 0.005 M cysteine, 1.1 g of wet cells, 1.2 mmoles of fructose, and 1 mmole of sodium bicarbonate as indicated. Fermentation was for 11 hr.

[b] Values expressed in millimoles.

TABLE 2. *Distribution of radioactivity in acetate formed during fermentation of fructose by Clostridium formicoaceticum in the presence of* ^{14}C*-carbon dioxide*[a]

Time (hr)	CH_3COOH	CH_3—	—COOH	CH_3/COOH
1	840	460	465	1.0
3	3,580	2,070	1,410	1.4
19.5	11,320	6,070	6,310	0.96

[a] Values are expressed in counts per minute per millimole. Reaction vessels contained 2 mmoles of ^{14}C-potassium bicarbonate (specific activity = 300,000 counts per min per mmole), 2 mmoles of fructose, and 3 g of wet cells in 40 ml of 0.1 M potassium phosphate, *p*H 7.0, with 0.005 M cysteine.

DISCUSSION

The results presented clearly show that *C. thermoaceticum* and *C. formicoaceticum* are very similar metabolically. Both bacteria are strict anaerobes which ferment hexoses almost stoichiometrically to 3 moles of acetate. The fermentation is via the Embden Meyerhof pathway. In both organisms, CO_2 acts as electron acceptor and is reduced to acetate by a pathway most likely involving formate, derivatives of tetrahydrofolate, and a methylcorrinoid.

Although the organisms are very similar, they differ in several aspects. First, *C. formicoaceticum* is a mesophile growing best at about 37 C (1, 5), whereas *C. thermoaceticum* is a thermophile with an optimum growth temperature of ca. 60 C (6). Second, the organisms are different in their requirement for the hexose substrate. *C. formicoaceticum* grows on fructose but not on glucose (1). *C. thermoaceticum*, on the other hand, is able to use both these sugars as well as several other hexoses (6). Third, a difference has been observed regarding the specificity for NAD and NADP of enzymes isolated from the two organisms. Thus, formate dehydrogenase and 5,10-methylenetetrahydrofolate dehydrogenase from *C. formicoaceticum* are specific for NAD, whereas the same enzymes from *C. thermoaceticum* are specific for NADP (14; W. E. O'Brien, L. Ljungdahl, and J. M. Brewer, Fed. Proc. p. 873, 1971). It would be of interest to examine other NAD- or NADP-requiring enzymes from the two organisms regarding the specificity for

TABLE 3. *Fermentation of labeled fructose by cells of Clostridium formicoaceticum*[a]

Substrate	Initial fructose	CO_2	CH_3COOH	CH_3	COOH
Fructose-*1*-^{14}C	16,280	169	4,950	4,500	86
Fructose-*1*-^{14}C + HCO_3^-	16,280	72	4,560	5,140	55
Fructose-*6*-^{14}C	16,450	108	4,100	4,400	31
Fructose-*6*-^{14}C + HCO_3^-	16,450	32	5,460	5,100	39
Fructose-*4*-^{14}C	1,670	432	197	60	98
Fructose-*4*-^{14}C + HCO_3^-	1,670	244	123	53	52
Fructose-*5*-^{14}C	6,100	62	1,357	72	1,365
Fructose-*5*-^{14}C + HCO_3^-	6,100	5	1,120	15	1,190

[a] Values are expressed in counts per minute per millimole. Each incubation contained 20 ml of the cell suspension, 1.2 mmoles of fructose, and 1 mmole of sodium bicarbonate, as indicated.

TABLE 4. *Conversion of 5-methyltetrahydrofolate and methylcobalamin into acetate by cell-free extracts of Clostridium formicoaceticum*

Substrate[a]	Time (min)	Substrate		Acetate formed	
		nmoles	dpm	nmoles	dpm
$^{14}CH_3$-B_{12}	20	250	55,000	56	12,300
	40	250	55,000	100	22,100
$^{14}CH_3$-THF	10	74	49,000	11	7,380
	20	74	49,000	19	12,400
$^{14}CH_3$-THF – pyruvate	20	74	49,000		630

[a] Reactions contained sodium pyruvate, 30 μmoles; potassium phosphate (*p*H 7.0), 50 μmoles; ferrous ammonium sulfate, 3 μmoles; cysteine, 2 μmoles; 5-methyltetrahydrofolate ($^{14}CH_3$THF) or methylcobalamin ($^{14}CH_3$-B_{12}), as indicated, and cell-free extract (15 mg of protein) in a volume of 1 ml.

TABLE 5. *Activities in an extract of Clostridium formicoaceticum of enzymes involved in conversion of carbon dioxide into 5-methyltetrahydrofolate*

Enzyme	Units/mg of protein[a]
Formate dehydrogenase (NAD)[b]	0.006
Formate dehydrogenase (MV)[b]	0.15
10-Formyltetrahydrofolate synthetase	8.7
5,10-Methenyltetrahydrofolate cyclohydrolase	0.15
5,10-Methylenetetrahydrofolate dehydrogenase	8.1
5,10-Methylenetetrahydrofolate reductase	0.016

[a] Units are given as micromoles of substrate used or product formed per min per milligram of protein. The extracts contained 23.1 mg of protein per ml.

[b] Nicotinamide adenine dinucleotide (NAD) or methyl viologen (MV) was the electron acceptor, as indicated.

the two coenzymes. Fourth, the patterns of labeling of acetate formed from $^{14}CO_2$ are different in the two organisms. With *C. thermoaceticum* it has been observed that more $^{14}CO_2$ is incorporated into the carboxyl carbon of acetate compared with the methyl carbon (2, 31). Similar results were obtained by Wood (31) by using $^{13}CO_2$ and mass spectrometry. He found that one-third of the acetate was synthesized completely from CO_2 and that one-third was labeled only in the carboxyl group. The rest of the acetate was unlabeled. Wood (31) also showed that *C. thermoaceticum* catalyzes an exchange between the carboxyl group of acetate and CO_2 and postulated that this exchange may account for the higher incorporation of $^{14}CO_2$ into the carboxyl group. With *C. formicoaceticum*, the acetate formed during fermentation in the presence of $^{14}CO_2$ always has been equally labeled in both carbon atoms. Furthermore, in experiments with $^{13}CO_2$ and mass spectrometry, Schulman et al. (26) found that about one-third of the acetate was totally synthesized from CO_2 and that the rest of the acetate was unlabeled. Thus, no singly labeled acetate was formed by *C. formicoaceticum*. Consequently, it appears that *C. formicoaceticum* does not catalyze an exchange between CO_2 and the carboxyl group of acetate.

The mechanism of total synthesis of acetate in heterotrophic bacteria has been largely unknown. Only recently has evidence been obtained which shows that formate, tetrahydrofolate derivatives, and a methylcorrinoid are

involved in the formation of the methyl group of acetate. Extracts of *C. formicoaceticum* contain all enzymes necessary for the formation of the 5-methyl group of 5-methyltetrahydrofolate from CO_2, and they convert 5-methyltetrahydrofolate as well as methyl-B_{12} to acetate. These findings are strong evidence for a pathway of acetate synthesis in *C. formicoaceticum* similar to that postulated for *C. thermoaceticum*. However, with both organisms, it remains to establish that the methyl group of 5-methyltetrahydrofolate is transferred to a corrinoid. The mechanism of carboxylation of the methylcorrinoid is also still unknown. These problems must be solved before the pathway of total synthesis of acetate from CO_2 is rigidly established.

Recently, Parker et al. (22) demonstrated with *C. thermoaceticum* that 5-methyltetrahydrofolate becomes highly labeled during pulse-labeling experiments with $^{14}CO_2$ and that it has the properties of an intermediate in the synthesis of acetate from CO_2. Such experiments were used earlier to demonstrate that methylcorrinoids likely are intermediates (18). Parker and Wood (*personal communication*) also have found 5,10-methylenetetrahydrofolate reductase in extracts of *C. thermoaceticum*.

The equilibrium of the reaction catalyzed by formate dehydrogenase is far toward CO_2 with pyridine nucleotides as electron donors, and it has been questioned whether this enzyme can be responsible for the reduction of CO_2 to formate (14). This obstacle is now removed. Recently, Jungermann et al. (9) demonstrated that formate dehydrogenase from *C. pasteurianum* can catalyze the reduction of CO_2 to formate with reduced ferredoxin when the enzyme is coupled with 10-formyltetrahydrofolate synthetase. Similarly, with the enzyme from *C. thermoaceticum*, CO_2 is reduced by NADPH or reduced methyl viologen when it is coupled with 10-formyltetrahydrofolate synthetase (J. R. Andreesen and L. G. Ljungdahl, Bacteriol. Proc., p. 257, 1971). The formation of formate also has been observed with reduced methyl viologen with the enzyme from *C. formicoaceticum* (Andreesen, *unpublished data*).

The demonstration that *C. formicoaceticum*, like *C. thermoaceticum*, is capable of synthesizing acetate from CO_2 through a pathway involving formate and tetrahydrofolate derivatives shows that this pathway is used in nature for a net fixation of CO_2. Clearly, it is possible that it can account for fixation of CO_2 in other organisms as well; one possible example is the incorporation of CO_2 into methionine, as has been demonstrated by Jungermann et al. (10).

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LITERATURE CITED

1. Andreesen, J. R., G. Gottschalk, and H. G. Schlegel. 1970. *Clostridium formicoaceticum* nov. spec. Isolation, description, and distinction from *C. aceticum* and *C. thermoaceticum*. Arch. Microbiol. **72**:154-174.
2. Barker, H. A., and M. D. Kamen. 1945. Carbon dioxide utilization in the synthesis of acetic acid by *Clostridium thermoaceticum*. Proc. Nat. Acad. Sci. U.S.A. **30**:219-225.
3. Blair, J. A., and K. J. Saunders. 1970. A convenient method for the preparation of DL-5-methyltetrahydrofolic acid (DL - 5 - methyl - 5,6,7,8 - tetrahydropteroyl-L-monoglutamic acid). Anal. Biochem. **34**: 376-381.
4. Donaldson, K. O., and J. C. Keresztesy, 1961. Further evidence on the nature of prefolic A. Biochem. Biophys. Res. Commun. **5**:289-292.
5. El Ghazzawi, E. 1967. Neuisolerung von *Clostridium aceticum* Wieringa und stoffwechselphysiologische Untersuchungen. Arch. Mikrobiol. **57**:1-19.
6. Fontaine, F. E., W. E. Peterson, E. McCoy, M. J. Johnson, and G. J. Ritter. 1942. A new type of glucose fermentation by Clostridium thermoaceticum n. sp. J. Bacteriol. **43**:701-715.
7. Ghambeer, R. K., H. G. Wood, M. Schulman, and L. Ljungdahl. 1971. Total synthesis of acetate from CO_2. III. Inhibition by alkylhalides of the synthesis from CO_2, methyltetrahydrofolate and methyl-B_{12} by *Clostridium thermoaceticum*. Arch. Biochem. Biophys. **143**:471-484.
8. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**:751-766.
9. Jungermann, K., H. Kirchniawy, and R. K. Thauer. 1970. Ferredoxin dependent CO_2 reduction to formate in *Clostridium pasteurianum*. Biochem. Biophys. Res. Commun. **41**:682-689.
10. Jungermann, K., R. K. Thauer, and K. Decker. 1968. The synthesis of one-carbon units from CO_2 in *Clostridium kluyveri*. Eur. J. Biochem. **3**:351-359.
11. Katzen, H. M., and J. M. Buchanan. 1965. Enzymatic synthesis of methyl group of methionine. VIII. Repression-derepression, purification, and properties of 5,10-methylenetetrahydrofolate reductase from *Escherichia coli*. J. Biol. Chem. **240**:825-835.
12. Koehler, L. H. 1952. Differentiation of carbohydrates by anthrone reaction rate and color intensity. Anal. Chem. **24**:1576-1579.
13. Kornblatt, J. A., P. Bernath, and J. Katz. 1964. The determination of specific activity of $BaC^{14}O_3$ by liquid scintillation assay. Int. J. Appl. Radiat. Isotop. **15**:191-194.
14. Li, L.-F., L. Ljungdahl, and H. G. Wood. 1966. Properties of nicotinamide adenine dinucleotide phosphate-dependent formate dehydrogenase from *Clostridium thermoaceticum*. J. Bacteriol. **92**:405-412.
15. Linke, H. A. B. 1969. Der Fructose-Stoffwechsel von *Clostridium aceticum*. Zentrabl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. 2 **123**:369-379.

16. Linke, H. A. B. 1969. CO_2-Fixierung durch *Clostridium aceticum*: $^{14}CO_2$-Kurzzeiteinbau und Pyruvatstoffwechsel. Arch. Mikrobiol. **64**:203–214.
17. Ljungdahl, L., J. M. Brewer, S. H. Neece, and T. Fairwell. 1970. Purification, stability, and composition of formyltetrahydrofolate synthetase from *Clostridium thermoaceticum*. J. Biol. Chem. **245**:4791–4797.
18. Ljungdahl, L., E. Irion, and H. G. Wood. 1965. Total synthesis of acetate from CO_2. I. Co-methylcobyric acid and Co(methyl)-5-methoxybenzimidazylcobamide as intermediates with *Clostridium thermoaceticum*. Biochemistry **4**:2771–2780.
19. Ljungdahl, L. G., and H. G. Wood. 1969. Total synthesis of acetate from CO_2 by heterotrophic bacteria. Annu. Rev. Microbiol. **23**:515–538.
20. Ljungdahl, L., H. G. Wood., E. Racker, and D. Couri. 1961. Formation of unequally labeled fructose-6-phosphate by an exchange reaction catalyzed by transaldolase. J. Biol. Chem. **236**:1622–1625.
21. Mervyn, L., and E. L. Smith. 1969. Methylcobalamin [α(5,6-dimethylbenzimidasolyl)-Co-methylcobamide]. Biochem. Prep. **12**:129–130.
22. Parker, D. J., T. Wu, and H. G. Wood. 1971. Total synthesis of acetate from CO_2: methyltetrahydrofolate, an intermediate, and a procedure for separation of the folates. J. Bacteriol. **108**:770–776.
23. Phares, E. F. 1951. Degradation of labeled propionic and acetic acids. Arch. Biochem. **33**:173–178.
24. Poston, J. M., K. Kuratomi, and E. R. Stadtman. 1966. The conversion of carbon dioxide to acetate. I. The use of cobalt-methyl cobalamin as a source of methyl groups for the synthesis of acetate by cell-free extracts of *Clostridium thermoaceticum*. J. Biol. Chem. **241**: 4209–4216.
25. Rabinowitz, J. C., and W. E. Pricer, Jr. 1962. Formyltetrahydrofolate synthetase. I. Isolation and crystallization of the enzyme. J. Biol. Chem. **237**:2898–2902.
26. Schulman, M., D. Parker, L. G. Ljungdahl, and H. G. Wood. 1972. Total synthesis of acetate from CO_2. V. Determination by mass analysis of the different types of acetate formed from $^{13}CO_2$ by heterotrophic bacteria. J. Bacteriol. **109**:633–644.
27. Swim, H. E., and L. O. Krampitz. 1954. Acetic acid oxidation by Escherichia coli: evidence for the occurrence of a tricarboxylic acid cycle. J. Bacteriol. **67**:419–425.
28. Trudinger, P. A. 1970. On the absorbancy of reduced methyl viologen. Anal. Biochem. **36**:222–225.
29. Uyeda, K., and J. C. Rabinowitz. 1967. Enzymes of clostridial purine fermentation. Methylenetetrahydrofolate dehydrogenase. J. Biol. Chem. **242**:4378–4385.
30. Van Slyke, D. D., and J. Folch. 1940. Manometric carbon determination. J. Biol. Chem. **136**:509–541.
31. Wood, H. G. 1952. A study of carbon dioxide fixation by mass-determination of the types of C^{13}-acetate. J. Biol. Chem. **194**:905–931.
32. Wood, H. G. 1952. Fermentation of 3,4-C^{14}- and 1-C^{14}-labeled glucose by *Clostridium thermoaceticum*. J. Biol. Chem. **199**:579–583.