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Acetate synthesis from 2 CO_2 in acetogenic bacteria: is carbon monoxide an intermediate?

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Abstract. Cultures of *Acetobacterium woodii* and *Clostridium thermoaceticum* growing on fructose or glucose, respectively, were found to produce small, but significant amounts of carbon monoxide. In the gas phase of the cultures up to 53 ppm CO were determined. The carbon monoxide production was completely inhibited by 1 mM cyanide. Cultures and cell suspensions of both acetogens incorporated ^{14}CO specifically into the carboxyl group of acetate. This CO fixation into C_1 of acetate was unaffected by cyanide (1 mM). The findings are taken to indicate that CO (in a bound form) is the physiological precursor of the C_1 of acetate in acetate synthesis from CO_2. The cyanide inhibition experiments support the hypothesis that the cyanide-sensitive carbon monoxide dehydrogenase may serve to reduce CO_2 to CO rather than to incorporate the carbonyl into C_1 of acetate.

Key words: CO_2 reduction to acetate – *Clostridium thermoaceticum* – *Acetobacterium woodii* – Carbon monoxide dehydrogenase – Carbon monoxide fixation

Introduction

Acetogenic bacteria catalyze the reduction of 2 CO_2 to acetate in their energy metabolism (for a recent review see Wood et al. 1982). The synthesis of the methyl group of acetate from CO_2 is known to proceed via formate, formyl tetrahydrofolate, and methyl tetrahydrofolate as intermediates. The formation of the carboxyl group of acetate, however, remained obscure until recently. Schulman et al. (1973) demonstrated with cell extracts of glucose-grown *Clostridium thermoaceticum* that C_1 of pyruvate rather than free CO_2 serves as the carboxyl donor for acetate synthesis.

Acetogenic bacteria contain high activities of a carbon monoxide dehydrogenase, which in vitro catalyzes the oxidation of CO with methyl viologen as artificial electron acceptor (Diekert and Thauer 1978; Diekert and Ritter 1982; Sharak-Genthner and Bryant 1982; Lynd et al. 1982; Clark et al. 1982). An enzyme system purified from *C. thermoaceticum* and containing the CO dehydrogenase was shown to convert ^{14}CO, [$^{12}CH_3$]tetrahydrofolate and CoA to acetyl CoA, which was labeled in the C_1 position of the acetate (Hu et al. 1982). It was proposed that CO dehydrogenase in vivo may serve to incorporate a bound formyl derivative formed from CO_2 or C_1 of pyruvate into acetate.

Recently we demonstrated that autotrophically growing *Acetobacterium woodii* incorporated ^{14}CO specifically into C_1 of acetate, and we observed an isotopic exchange of CO_2 and CO (Diekert and Ritter 1983). The data were taken to suggest that CO might be an intermediate in acetate formation from CO_2 and that CO dehydrogenase might provide the carbon monoxide by CO_2 reduction (Fig. 1). The low rates of CO incorporation into acetate and of label exchange between CO_2 and CO suggested that a bound carbonyl, rather than free carbon monoxide, is formed from CO_2 and is the source of C_1 of acetate, and that the exchange between bound and free CO is slow with respect to the rate of the overall reaction (Fig. 1). Thus, according to our working hypothesis, the mechanism of acetate formation from methyl tetrahydrofolate and CO_2 would be very closely related to that of the chemical synthesis of acetate from CO and methanol (Monsanto process or Reppe process; for literature see Mullen 1980). This type of carbonylation reaction would be mechanistically different from a formylation reaction as proposed by Hu et al. (1982).

Cyanide was shown to inactivate CO dehydrogenase of acetogenic bacteria (Diekert and Thauer 1978). This finding allows one to test the scheme proposed in Fig. 1, which, if correct, predicts the following: (1) Free CO should be formed in low amounts as a side product of acetate formation from 2 CO_2. (2) Formation of CO and of acetate from CO_2 should be inhibited by cyanide. (3) Incorporation of CO into C_1 of acetate should not be affected by cyanide. We report here experiments that confirm our hypothesis with respect to these predictions.

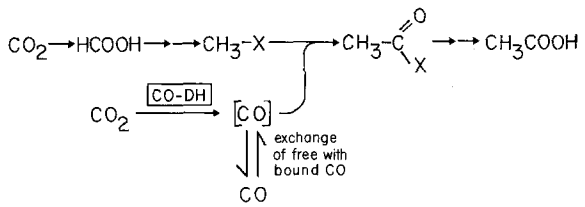

Fig. 1. Tentative scheme for the synthesis of acetate from 2 CO_2 catalyzed by acetogenic bacteria (Diekert and Ritter 1983). The CO dehydrogenase (= CO-DH) (Diekert and Thauer 1978) and the CO_2 reductase (Kearny and Sagers 1972) are known to be inactivated by cyanide

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Material and methods

Source of materials. $NaH^{14}CO_3$ (2.1×10^6 Bq/μmol) and Na[^{14}C]formate (1.9×10^6 Bq/μmol) were purchased from Amersham Buchler (Braunschweig, FRG). All gases and gas mixtures were from Messer Griesheim (Düsseldorf, FRG). *Acetobacterium woodii* strain WB 1 (DSM 1030) and *Clostridium thermoaceticum* (DSM 521) were obtained from the Deutsche Sammlung von Mikroorganismen (Göttingen, FRG).

Growth of the bacteria. A. woodii was grown at 30° C on either H_2/CO_2 (80:20, 200 kPa) or on fructose under N_2/CO_2 (80:20, 150 kPa), the media used were according to Diekert and Ritter (1982, 1983). *C. thermoaceticum* was grown in stoppered glass flasks on glucose medium (Diekert and Thauer 1978) at 55° C under 100% CO_2 (150 kPa or, where indicated, 110 kPa).

Preparation of cell suspensions. For the experiments performed with cell suspensions glucose-grown *C. thermoaceticum* and H_2/CO_2-grown *A. woodii* were used. The cells were harvested in the late logarithmic growth phase by centrifugation under N_2 for 30 min at 13,000 × g. The supernatant was removed and the cell pellet was washed with 50 mM potassium phosphate buffer pH 7.7 containing 50 mM 2-mercaptoethanol. Then the cells were resuspended in the same buffer. The final cell concentration was 20 mg/ml (dry weight) for *C. thermoaceticum* and 5 mg/ml for *A. woodii*. For the cyanide inhibition experiments the cells were preincubated 45 min at 30° C in the presence of cyanide (1 mM). The preparation of the cell suspensions was performed throughout under strictly anaerobic conditions.

Labelling experiments in cell suspensions. The labelling experiments were carried out in 56 ml serum-bottles with 9 ml of an assay mixture containing 50 mM potassium phosphate buffer pH 7.7 plus sodium formate, sodium pyruvate, 2-mercaptoethanol, 0.45 mmol each, 2 μg/ml resazurin, 50–100 μM sodium dithionite, and, where indicated, 1 mM potassium cyanide. The gas phase was H_2/CO_2 (80:20, 120 kPa) plus 0.35 ml ^{12}CO or ^{14}CO (2,300 Bq/μmol; final concentration in the gas phase 0.63%). The experiment was started by the injection of 1 ml cell suspension. The assay was then incubated for 15 min at 30° C (*A. woodii*) or at 55° C (*C. thermoaceticum*) under continuous shaking. Then the experiment was stopped by the injection of 1 ml 3 M perchloric acid.

Preparation and determination of ^{14}CO. ^{14}CO was prepared from ^{14}C-formate as follows: An 8 ml stoppered serum-bottle containing 2 ml 98% H_2SO_4 was repeatedly evacuated and gassed with N_2 and was then preincubated at 80° C. After the addition of 367,000 Bq of ^{14}C-formate (1.9×10^6 Bq/μmol) the solution was stirred at 80° C for 45 min. The gas phase was then quantitatively transferred to a second stoppered 8 ml serum-bottle, which had previously been evacuated. To this bottle 2.5 ml 1 M NaOH plus 50 mg $Na_2S \times 9\ H_2O$ were added. The bottle was shaken at room temperature for at least 6 h. The NaOH/sulfide solution served to remove a major part of contaminating $^{14}CO_2$ and oxygen. From the gas phase of the serum-bottle samples were withdrawn with a gas-tight syringe and added to the cultures or assay mixtures.

The CO concentration in the gas phase was determined either with a CO analyzer based on the HgO-to-Hg vapor conversion technique (Seiler et al. 1980) or by flame ionization detection after reduction of CO to CH_4 at 380° C on a nickel catalyst (Methanizer; supplied by Varian, Darmstadt, FRG). The label of ^{14}CO was determined as described earlier (Diekert and Ritter 1983). Samples were taken from the gas phase containing ^{14}CO, and $^{14}CO_2$ and oxygen were removed with a NaOH/sulfide solution. The latter was repeated twice until no more label could be detected in the alkaline solution. The gas phase was then transferred to a serum-bottle containing a partially purified CO dehydrogenase from *C. thermoaceticum* and excess methyl viologen. The serum-bottle was incubated several hours at 55° C, until all of the CO had been oxidized to CO_2. The $^{14}CO_2$ was determined after acidification of the enzyme mixture and trapping the $^{14}CO_2$ by NaOH.

Isolation and determination of $^{14}CO_2$, acetate, formate, and pyruvate. Acetate, formate, and pyruvate were isolated using the following procedure: 5 ml aliquots withdrawn from the cell suspensions were adjusted to pH 8 by the addition of KOH. After incubation for 1 h at 0° C the samples were centrifuged at 27,000 × g for 15 min. Two milliliter of the supernatant were applied to a column (∅ 4 mm) containing 2 g of DOWEX 1 × 8 resin, 100–200 mesh, formate as counterion. The column was washed with 10 ml H_2O. Acetate was eluted with 20 ml 50 mM HCOOH in 2 ml fractions; the label of [^{14}C]acetate was recovered in fractions 5–9. Then formate was eluted in 2 ml fractions with 14 ml 1 M HCOOH, the formate being detected in fractions 5–7. Finally, pyruvate could be eluted with 4 M HCl and was recovered in fractions 2–5 (2 ml fractions). 0.2 ml of each fraction was counted for radioactivity.

The acetate-containing fractions were pooled and adjusted to pH 8 with KOH. They were flash evaporated to dryness and the residue was dissolved in 1.2 ml H_2O. One ml of the solution was then dried in a desiccator above P_4O_{10} and the acetate was degraded using the method of Simon and Floss (1967). The CO_2 formed from C_1 of acetate was trapped in NaOH. The label present in the methyl group was detected as $^{14}CO_2$ after the oxidation of the methyl amine (= C_2 of acetate) by $KMnO_4$.

Acetate (Dorn et al. 1978) and pyruvate (Passonneau and Lowry 1970) were determined enzymatically. Formate was assayed according to Lang and Lang (1972). CO_2 was determined gas chromatographically. $^{14}CO_2$ was trapped in NaOH from samples taken from the gas phase and the radioactivity was measured by liquid scintillation counting (Diekert and Ritter 1983).

Results

CO formation and CO fixation by growing acetogenic bacteria. Clostridium thermoaceticum was grown on glucose medium at 55° C in the dark. The CO concentration in the gas phase was determined gas chromatographically. The cultures were found to produce small amounts of carbon monoxide (Fig. 2): in the gas phase up to 53 $\mu l \cdot l^{-1}$ could be detected. The CO concentration was observed to increase up to a maximum and then to decrease again at the end of the logarithmic growth phase (Fig. 2). Cultures of *Acetobacterium woodii* growing on fructose or on H_2/CO_2 (80:20) also produced carbon monoxide, however, the

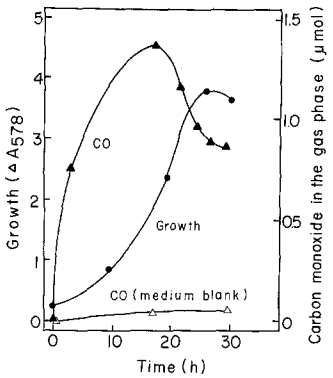

Fig. 2. Production of carbon monoxide (▲) by a culture of *Clostridium thermoaceticum* during growth (●) on glucose medium. The culture volume was 240 ml, the gas phase (320 ml) was 100% CO_2 (150 kPa). 1 μmol CO in the gas phase corresponded to a CO concentration of 47 ppm, a ΔA_{578} of 1 to a cell concentration of 300 mg (dry weight) per l. The cells were grown at 55° C in the dark. As a blank uninoculated medium was incubated under the same conditions

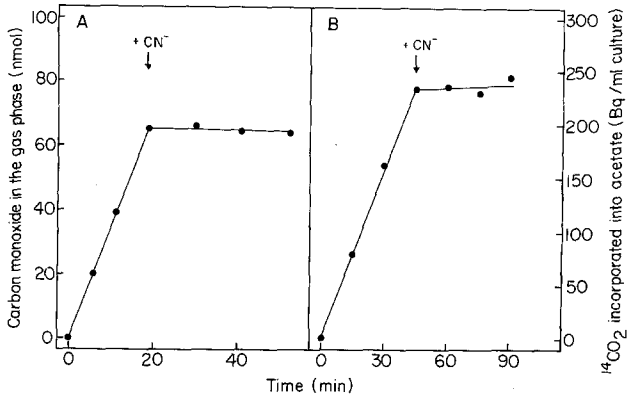

Fig. 3A, B. Effect of cyanide on CO formation **A** and $^{14}CO_2$ incorporation into acetate **B** in *Acetobacterium woodii* growing on H_2/CO_2 (80:20; 200 kPa; 400 ml gas phase). The 120 ml culture was incubated in the dark under continuous shaking. The cell concentration was about 150 mg (dry weight) per l. 1 μmol of CO in the gas phase corresponded to a CO concentration of 37 ppm. Experiment **B** was started by the addition of $NaH^{14}CO_3$ (1.5×10^6 Bq) to the culture. At the time indicated by the arrow 1 mM cyanide was added

Table 1. Fixation of ^{14}CO into the carboxyl group of acetate during growth of *Clostridium thermoaceticum* on glucose and CO. For the experiment a 60 ml culture with 70 ml gas phase (100% CO_2, 110 kPa) was used. The experiment was started by the injection of 3.8 ml ^{14}CO (1530 Bq/μmol; final concentration in the gas phase 5%). At $t = 0$ h the glucose concentration was 94.1 mM, at $t = 6$ h 87.3 mM; the acetate concentration was at $t = 0$ h 15.5 mM, at $t = 6$ h 34.0 mM. The $^{14}CO_2$ initially present was due to a contamination of the ^{14}CO with $^{14}CO_2$

Time after addition of ^{14}CO (h)	CO (μmol) in the gas phase	Total radioactivity (Bq) of			
		^{14}CO	$^{14}CO_2$	^{14}C acetate	C_1 of acetate
0	170	261,000	1,500	0	0
6	31	46,920	129,000	50,330	46,775

maximal CO concentration measured in the gas phase was approximately 10-fold lower than in *C. thermoaceticum* cultures. Medium blanks (Fig. 2) as well as cultures of *Wolinella succinogenes* growing on formate and fumarate did not generate CO in detectable amounts.

Growth of *C. thermoaceticum* on glucose in the presence of $^{14}CO_2$ was accompanied by the conversion of $^{14}CO_2$ to ^{14}CO, as has been observed in cultures of *A. woodii* (Diekert and Ritter 1983). This finding indicates that CO_2 may be the source of CO production. It could not, however, be excluded that the ^{14}CO formation from $^{14}CO_2$ might have proceeded via a product of CO_2 fixation such as acetate or via a previous exchange of the label into an intermediate of metabolism, for instance into C_1 of pyruvate.

Both acetogenic bacteria mediated a fixation of ^{14}CO specifically into the carboxyl group of acetate (Table 1; Diekert and Ritter 1983). The cultures converted 63% of the ^{14}CO to $^{14}CO_2$, and only about 20% of the CO was directly incorporated into C_1 of acetate. *A. woodii* oxidized under comparable conditions less than 10% of the CO to CO_2 (Diekert and Ritter 1983).

Cyanide inhibition of CO and acetate formation from CO_2 and H_2 in growing Acetobacterium woodii. When cyanide (1 mM) was added to cultures of *A. woodii* growing on H_2/CO_2 (80:20), CO formation ceased at once (Fig. 3A). After evacuation and gassing of the culture with H_2/CO_2, no more CO production was observed. A control culture incubated in the absence of cyanide started CO formation again immediately after removal of the CO already generated. When ^{14}CO (400 nmol, corresponding to 15 ppm in the gas phase) was added to the cyanide treated culture, the CO concentration in the gas phase slowly decreased linearly with time; the label was then recovered exclusively in C_1 of acetate rather than in CO_2.

The effect of cyanide on acetate formation from $^{14}CO_2$ was investigated under the same growth conditions. Before the addition of cyanide, $^{14}CO_2$ was readily incorporated into acetate. Cyanide (1 mM) almost completely inhibited CO_2 fixation into acetate (Fig. 3B). Schmidt degradation of the acetate revealed that the incorporation of $^{14}CO_2$ into both C-atoms was affected by cyanide. The ratio of the radioactivity in C_1 and C_2 remained constant at 50%/50% during the incubation period in the presence of cyanide.

The effect of cyanide on acetate formation from CO_2, CO, and formate in cell suspensions. The effect of cyanide (1 mM) on [^{14}C]acetate formation from $^{14}CO_2$ was investigated also in cell suspensions of *A. woodii*. Cyanide inhibited the conversion of CO_2 to acetate, formate, and CO. The formation of acetate from $^{14}CO_2$ was affected to the same extent as the exchange of $^{14}CO_2$ and ^{12}CO, when the cells were incubated with formate (50 mM) and CO (0.63% in the gas phase). The cyanide inhibition of formate formation from CO_2 is

Table 2. The effect of cyanide on the incorporation of ^{14}CO (15.6 μmol corresponding to a final concentration of 0.63% in the gas phase; specific radioactivity 2300 Bq/μmol) into acetate by cell suspensions of *Clostridium thermoaceticum* and *Acetobacterium woodii*. For experimental details see material and methods section

Organism	[KCN] (mM)	^{14}C (Bq) incorporated into acetate	
		$-COOH$	$-CH_3$
C. thermoaceticum	0	423	<13
	1	786	0
A. woodii	0	482	<10
	1	639	0

not surprising, because CO_2 reductase is known to be inactivated by cyanide (Kearny and Sagers 1972).

When labeled carbon monoxide (instead of $^{14}CO_2$) and [^{12}C]formate were added to the cell suspensions, the incorporation of radioactivity into acetate was not affected, but even seemed to be slightly stimulated by cyanide (Table 2). The label was recovered exclusively in C_1 of acetate. Similar results were obtained with cell suspensions of *C. thermoaceticum* (Table 2). The findings are consistent with our observations on the effect of cyanide on ^{14}CO incorporation into acetate in growing cultures of *A. woodii*.

The experiments with the cell suspensions of *A. woodii* were all carried out in the presence of 50 mM pyruvate, because pyruvate turned out to be required both for $^{14}CO_2$ and ^{14}CO fixation into acetate. Less than 10% of the pyruvate added was converted to acetate by the cell suspensions; however, $^{14}CO_2$ and ^{14}CO were not incorporated into pyruvate under the same experimental conditions. These findings argue against the role of pyruvate as the intermediate carboxyl donor for acetate formation from CO_2 in *A. woodii*. The role of pyruvate in total synthesis of acetate from CO_2 and CO is not yet understood and will require further investigation.

Discussion

In the present communication we report that acetogenic bacteria during growth on glucose, fructose, or H_2 and CO_2 produced significant amounts of carbon monoxide. This finding is consistent with the hypothesis that CO is an intermediate in CO_2 reduction to acetate. The low amount of carbon monoxide formed during growth can best be explained by some thermodynamic considerations. Assuming that CO_2 is the substrate for CO generation, CO is formed during growth on H_2 and CO_2 according to the following equation:

$$CO_2 + H_2 \rightarrow CO + H_2O \; (\Delta G^{\circ\prime} = +20 \text{ kJ/mol}).$$

This reaction is endergonic under standard conditions (CO_2, H_2, and CO in the gaseous state; temperature 25° C) (Thauer et al. 1977). From the $\Delta G^{\circ\prime}$ value and a gas phase consisting of 20% CO_2 and 80% H_2 at 100 kPa it can be calculated that at most 50 ppm CO (equilibrium concentration) in the gas phase can be expected. Also it must be considered that, according to our hypothesis, CO is an intermediate and is therefore consumed in a subsequent reaction. If this is valid, the concentration of CO detectable in the gas phase could be much lower than the calculated equilibrium concentration.

It can also be calculated that at higher temperatures (55° C) the equilibrium concentration of CO should be higher. This would be an explanation for the higher CO concentrations found in the gas phase of *Methanobacterium thermoautotrophicum* cultures (Conrad and Thauer 1983), which grow at 65° C. In this methanogen CO has also been proposed to be an intermediate in CO_2 reduction to acetyl CoA (Stupperich et al. 1983; Conrad and Thauer 1983), which is the first CO_2 fixation product in the anabolism of the methanogens (Fuchs and Stupperich 1980; Stupperich and Fuchs 1983). These bacteria also contain a CO dehydrogenase, which was suggested to mediate the formation of CO from CO_2 as the first step of CO_2 reduction to C_1 of acetyl CoA (Stupperich and Fuchs 1983).

We have shown here that CO production, but not CO incorporation into acetate, was cyanide-sensitive in acetogens. These data are consistent with our working hypothesis as outlined in Fig. 1 and favor the interpretation that CO, formed via CO dehydrogenase, is the precursor of the acetate carboxyl group. In the growing cultures, however, free CO was incorporated only at very low rates (about 0.1 nmol/min and mg of protein) into acetate. This observation led to the conclusion that CO is a bound intermediate in acetate synnthesis from CO_2 and that the rate of exchange between free and bound CO is only very slow (Diekert and Ritter 1983; see also Fig. 1).

CO incorporation into acetate by cell suspensions of *A. woodii* was strictly dependent on pyruvate. The $^{14}CO_2$ labelling studies performed with autotrophically grown cells of *A. woodii* showed that in this organism C_1 of pyruvate is probably not the precursor of the acetate carboxyl group as proposed for heterotrophically grown *C. thermoaceticum* (Schulman et al. 1973). This was also shown by pyruvate labeling experiments in cultures of *A. woodii* (Eden and Fuchs 1982). Whether pyruvate serves different functions in *A. woodii* and *C. thermoaceticum* will require further investigation.

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