JOURNAL OF BACTERIOLOGY, Apr. 1985, p. 67–71
0021-9193/85/040067-05$02.00/0

Vol. 162, No. 1

Inhibition of Methanogenesis and Carbon Metabolism in *Methanosarcina* sp. by Cyanide

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Received 24 October 1984/Accepted 10 January 1985

NaCN was tested for its inhibitory effects on growth of and metabolism by *Methanosarcina barkeri* 227. NaCN (10 μM) inhibited catabolism of acetate methyl groups to CH_4 and CO_2 but did not inhibit methanogenesis from methanol, CO_2, methylamine, or trimethylamine. NaCN also inhibited the assimilation of methanol or CO_2 (as the sole carbon source) into cell carbon and stimulated the assimilation of acetate. These results suggest that inhibition by NaCN was a result of its action as an inhibitor of in vivo CO dehydrogenase. The results also implicate CO dehydrogenase in the oxidation of acetate but not methanol methyl groups to CO_2.

Methane is generated from acetate in *Methanosarcina* spp. by a decarboxylation reaction in which the methyl group is reduced to CH_4 and the carboxyl group is oxidized to CO_2 (1, 21). This reaction, which may involve coenzyme M (2-mercaptoethanesulfonate) as an intermediate, generates energy for ATP synthesis and growth by an unknown mechanism (2, 20–22). The free energy yield from the aceticlastic reaction (-36 kJ mol^{-1}) is insufficient for the formation of 1 mol of ATP per mol of acetate cleaved (21). Electron transport phosphorylation is indicated for ATP synthesis (26).

Cyanide was first reported to inhibit methanogenic bacteria growing on acetate (11, 21). Growth inhibition is probably directed specifically toward catabolism because growth and methanogenesis on H_2-CO_2 or methanol are not inhibited by cyanide (7, 25; M. R. Smith, M. Hart, and M. Weiss, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, I7, p. 140). Cyanide is a well-known inhibitor of cytochromes, and methanogens that catabolize methyl groups were reported to possess cytochromes (6, 14, 16). However, cyanide is not specific in its action, and its general effects in vivo on the metabolism of methanogens have not been reported.

In this paper, we report that NaCN at low concentrations is a specific inhibitor of methanogenesis from acetate, oxidation of acetate methyl groups to CO_2, and assimilation of one-carbon units. Cyanide showed no selective activity on methyl-group oxidation from methanol. (Part of this work was reported previously [Smith et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1983.])

MATERIALS AND METHODS

Bacterial strain and culture media. *Methanosarcina barkeri* 227 (DSM 1538) was described previously (21). Cultures were maintained by transferring them in 50 ml of MOPS (morpholinepropanesulfonic acid) complex basal medium (complex medium) (20) in 160-ml serum bottles containing H_2-CO_2 (80:20; 202 kPa), methanol (40 mM), methylamine (25 mM), trimethylamine (25 mM), or sodium acetate (50 mM) as the only energy source. Complex medium also contained 0.2% yeast extract and 0.2% Trypticase (BBL Microbiology Systems, Cockeysville, Md.). Acetate was present in the medium at a 2 mM concentration because it was supplied by the Trypticase (22). Methanol cultures were also maintained on MOPS defined basal medium (defined medium) or PBBM medium (mineral medium) (12) for at least five transfers before they were used as inocula for experiments. The defined medium was identical to the complex medium except that 10 ml of Wolin vitamin solution (1) per liter and 1.0 ml of trace mineral (1) solution per liter were substituted for the yeast extract and Trypticase. Defined medium contained (grams $liter^{-1}$): NH_4Cl, 1.0; K_2HPO_4, 0.4; $MgCl_2 \cdot 6H_2O$, 0.1; MOPS, 2.62; NaOH, 0.3; resazurin, 0.0001, and cysteine hydrochloride, 0.5. Wolin trace mineral and vitamin solutions (1 and 10 ml $liter^{-1}$, respectively) were added before the medium was boiled under N_2. Sterile anaerobic $Na_2S \cdot 9H_2O$ and $NaHCO_3$ were added to final concentrations of 0.01 and 0.1%, respectively, before inoculation. The defined medium differed from the mineral medium in having MOPS instead of phosphate as a buffering agent and in having cysteine hydrochloride added. The mineral medium contained no added organic compounds other than the vitamin solution.

Defined medium or complex medium was used for all growth experiments with methanol as the energy source. Mineral medium was used only for growth experiments with H_2-CO_2 as the energy source. All media were prepared anaerobically under N_2 with distilled water and reagent grade chemicals.

Chemicals. Coenzyme M was purchased from Sigma Chemical Co., St. Louis, Mo. Methylcoenzyme M was synthesized as described previously (20). Bradford reagent (4) for protein assays was purchased from Bio-Rad Laboratories, Richmond, Calif.

Isotopes. [^{14}C]methanol (specific activity, 58 mCi $mmol^{-1}$), sodium [2-^{14}C]acetate (specific activity, 57.5 mCi $mmol^{-1}$), and sodium [1-^{14}C]acetate (specific activity, 54 mCi $mmol^{-1}$) were purchased from Amersham Corp., Arlington Heights, Ill.

Cell suspensions and extracts. Washed cell suspensions were prepared anaerobically in PD buffer (pH, 7.0) (20) from 50 ml of culture in complex medium. The suspension was distributed in 5-ml portions into 27-ml Balch tubes (1) inside an anaerobic glove box. Substrates, radioactive substrates,

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TABLE 1. Effect of cyanide on methane production by *M. barkeri* 227[a]

Substrate	Medium	Methane produced (μmol) with the following amount of NaCN (μM):					Days incubated
		0	1	10	100	1,000	
H_2-CO_2	Complex	1,109	720	1,304	84	6	3
Methylamine	Complex	835	761	751	114	4	7
Trimethylamine	Complex	1,089	1,072	925	355	3	7
Acetate	Complex	159	103	0	0	ND[b]	10
H_2-CO_2	Mineral	622	586	13	ND	3	5
H_2-CO_2	Mineral + acetate[c]	882	1,433	1,042	ND	9	4

[a] All cultures except those on 50 mM acetate were in 50 ml of medium in 160-ml serum bottles. Acetate cultures were grown in 10.5 ml of medium in 27-ml Balch tubes (1). Mineral medium was as described previously (12). Final substrate concentrations were 25 mM (methylamine or trimethylamine) or 50 mM (sodium acetate). H_2-CO_2 (80:20) was added at 202 kPa to media under an N_2 atmosphere. H_2-CO_2 cultures were shaken at 200 rpm.
[b] ND, Not determined.
[c] 10 mM.

and inhibitor solutions were then added to make the desired final concentrations from concentrated, anaerobic, filter-sterilized stock solutions. The suspensions were incubated in a 37°C water bath for 23 h.

Cell extracts were prepared from 10 liters of culture as described previously (20). Cells (8 to 10 g [wet weight]) were suspended in 5 to 8 ml of PD buffer and broken anaerobically under H_2 with a French press. The crude extracts were centrifuged at 30,000 × *g* for 20 min at 4 °C. The supernatant was collected anaerobically, and the cell pellet was discarded.

Experiments to determine the effects of NaCN on the rate of methanogenesis by resting cell suspensions were conducted with 56 μg of cell protein incubated at 37°C in 5-ml vials containing PD buffer and 10 mM sodium acetate or 10 mM methanol as described previously (20).

Growth. Growth was determined by observing changes in absorbance at 450 nm, using a Perkin-Elmer model LC-55 spectrophotometer. Absorbance determinations were possible with *M. barkeri* 227 because of its tendency to grow as finely dispersed particles. Cell yields at the end of incubation were determined by weight difference after filtering 20 to 40 ml of culture through preweighed 0.45-μm-pore-size polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.), washing the filters twice with 10 ml of distilled water, and then drying them to constant weight in a desiccator.

Growth rates were determined by observing absorbance at 450 nm and by calculating from the exponential increase in CH_4. The specific growth rate constant (μ) is the natural logarithm of 2 divided by the doubling time. Absorbance measurements and CH_4 measurements gave good agreement in the value of μ.

Methane. Methane and carbon dioxide were measured by gas chromatography as described previously (20–22). $^{14}CH_4$ and $^{14}CO_2$ were determined by constant volume sampling of the headspaces of cultures or cell suspensions followed by injection of the sample into an F&M gas chromatograph (with a thermoconductivity detector) connected in tandem to a Packard model 894 gas proportional counter (21). For analysis of $^{14}CH_4$ and $^{14}CO_2$ in cell suspensions, the suspensions were acidified before the headspace was sampled by adding 1 ml of 20 N H_2SO_4. The suspensions were shaken vigorously at intervals for at least 2 h before sampling to allow dissolved CO_2 to equilibrate with the headspace. Dissolved $^{14}CO_2$ was estimated by Henry's law. Methanogenesis by cell extracts was monitored at 15-min intervals with a Perkin-Elmer gas chromatograph and flame ionization detector (20).

Assays. The method of Bradford (4) was used to assay protein in cell extracts. Bovine serum albumin (fraction V; Sigma) was used as a standard. Cell protein in cell suspensions was assayed by the method of Hippe et al. (10). Methylcoenzyme M methylreductase activity and methanogenic activity from methanol in cell extracts were assayed in 5-ml vials by the method of Romesser and Balch (19), using the reaction mixtures and incubation conditions described previously (20).

Labeling experiments. The effects of NaCN on assimilation of carbon from ^{14}C-labeled substrates were determined for cultures growing in complex medium containing 40 mM methanol and 1 μCi of sodium [2-^{14}C]acetate, sodium [1-^{14}C]acetate, or [^{14}C]methanol. Cultures were inoculated (1% volume) into the medium and incubated at 35°C for 96 h. At the end of incubation, the cells were harvested by filtration onto 0.45-μm-pore-size Nuclepore membrane filters. Each filter was washed three times with 10 ml of cold 5% trichloroacetic acid and once with 10 ml of distilled water. The filters were then dried to constant weight in a desiccator and counted in Aquasol (New England Nuclear Corp., Boston, Mass.) in a liquid scintillation counter to determine the quantity of radioactive substrate assimilated.

Experiments to determine the effects of NaCN on the metabolism of radioactive methanol or acetate to gases in methanol-acetate mixtures employed acetate-grown cell suspensions prepared and distributed as described above. Methanol plus acetate was added to give a final concentration of 10 mM methanol and 1 mM sodium acetate. The tubes also received 2 μCi of [^{14}C]methanol or sodium [2-^{14}C]acetate and 89 μg of cell protein. Incubations were conducted at 37°C for 23 h. $^{14}CH_4$ and $^{14}CO_2$ were determined by gas chromatography and by counting with a Packard model 894 gas proportional counter connected in tandem to a gas chromatograph after acidification with 1 ml of 20 N H_2SO_4 (21). Sampling of headspaces for $^{14}CH_4$ and $^{14}CO_2$ was performed at 2 and 24 h after acidification to allow dissolved CO_2 to equilibrate with the headspace. The incubation mixtures were shaken vigorously at intervals during the equilibration period. No differences in $^{14}CH_4$ and $^{14}CO_2$ readings were observed at 2 and 24 h after acidification. Dissolved CO_2 was estimated by Henry's law.

RESULTS

Influence of medium composition and growth substrate on sensitivity to NaCN. Growth inhibition by NaCN varied with the energy source and composition of the medium (complex or mineral) (Table 1). Growth was estimated visually and from the quantity of methane produced at intervals during incubation at 35°C for the indicated times. NaCN inhibited methanogenesis of cultures in complex medium containing H_2-CO_2, methylamine, or trimethylamine only when the

NaCN concentration was 100 μM or greater (Table 1). Methanogenesis from acetate was more sensitive to cyanide than methanogenesis from the other substrates, with inhibition occurring at 10 μM NaCN.

Cultures in mineral medium (12) containing H_2-CO_2 as the growth substrate were inhibited by lower (10 μM) concentrations of NaCN than were H_2-CO_2 cultures in complex medium. Inhibition of growth on H_2-CO_2 in mineral medium did not occur if the medium was supplemented with 10 mM sodium acetate. This indicates that acetate overcame the metabolic block that was responsible for growth inhibition.

Cyanide exhibited the same pattern of inhibition on methanol cultures in defined medium as it did on H_2-CO_2 cultures in mineral medium (Table 2). Control cultures in defined medium containing 40 mM methanol but without NaCN grew with a doubling time of 14.1 h ($\mu = 0.049\ h^{-1}$). NaCN (10 μM) increased the doubling time to 21.7 h ($\mu = 0.032\ h^{-1}$), and 100 μM NaCN caused nearly complete inhibition of growth. In defined medium supplemented with 10 mM sodium acetate, the doubling time was 11.7 h ($\mu = 0.059\ h^{-1}$) and was unaffected by the addition of 10 μM NaCN. Final absorbances at a wavelength of 450 nm agreed with specific growth rate constant determinations.

Inhibition by NaCN of methanol and acetate assimilation into cell carbon. NaCN inhibited growth on H_2-CO_2 in mineral medium or on methanol in defined medium probably because it inhibited carbon assimilation. To determine if NaCN inhibition was caused by inhibition of methanol or CO_2 assimilation, cultures were inoculated into complex medium containing methanol and 1 μCi of radioactive methanol or acetate (Table 3). Complex medium and 10 μM NaCN were chosen because growth was not inhibited as in the previous experiments. Assimilation of [^{14}C]methanol, sodium [1-^{14}C]acetate, or sodium [2-^{14}C]acetate was compared in cultures incubated with and without 10 μM NaCN. The cultures grew exponentially during the 96 h of incubation.

Cultures in complex medium without added acetate produced 1,170 to 1,378 μmol of CH_4 and grew with doubling times of 10.3 to 10.8 h ($\mu = 0.067$ to $0.064\ h^{-1}$). The methyl and carboxyl carbon atoms of acetate were assimilated in approximately equimolar ratios (3.5 and 3.3 mmol g of cells^{-1}). About four times as much carbon was assimilated from methanol (14 mmol g of cells $^{-1}$) as from the methyl or carboxyl group of acetate. When 10 μM NaCN was present in the medium, greater quantities of acetate were assimilated (4.9 mmol g of cells^{-1}) and less methanol was assimilated (9.2 mmol g of cells^{-1}). The methyl and carboxyl carbon atoms of acetate continued to be assimilated in equal proportion. Specific growth rate constants, total methane, and final cell yields (based on absorbance at 450 nm) were not affected by the presence of 10 μM NaCN.

TABLE 2. Cyanide inhibition of growth of *M. barkeri* 227 on methanol in defined medium[a]

Addition	μ (CH_4) (h^{-1})	Final A_{450}	Total CH_4 (μmol)
None	0.049	0.156	341
10 μM NaCN	0.032	0.033	70
100 μM NaCN	0.002	0.006	9
10 mM sodium acetate	0.059	0.274	702
10 mM sodium acetate + 10 μM NaCN	0.059	0.352	846

[a] Defined medium is described in the text. Methanol (40 mM) was added as an energy source. The results are averages of duplicate determinations.

TABLE 3. Cyanide inhibition of acetate assimilation by *M. barkeri* 227 in complex medium[a]

Labeled substrate	NaCN (μM)	Total methane (μmol)	μ (h^{-1})	Labeled substrate assimilated (mmol g of cells^{-1})
[2-^{14}C]acetate	0	1,378	0.064	3.5
[1-^{14}C]acetate	0	1,170	0.067	3.3
$^{14}CH_3OH$	0	1,204	0.066	14.0
[2-^{14}C]acetate	10	1,267	0.063	4.9
[1-^{14}C]acetate	10	1,265	0.064	4.9
$^{14}CH_3OH$	10	1,233	0.064	9.2

[a] Complex medium is described in the text. Cultures (50 ml) were grown on 40 mM methanol in the presence of 1 μCi of [^{14}C]methanol or [^{14}C]acetate for 96 h at 37°C. Cells were then harvested by filtration, washed, dried, and counted. The specific growth rate constant (μ) was calculated from the exponential increase in methane. Results are averages of duplicate or triplicate determinations.

Effects of NaCN on the catabolism of methanol and acetate. Inhibition of growth on acetate in complex medium was probably a catabolic effect rather than an anabolic effect as occurred with H_2-CO_2 or methanol in mineral or defined medium. This was confirmed by testing the effect of 10 μM NaCN on the rate of methane production from acetate by washed cell suspensions of acetate-grown cells. The rate of methanogenesis from acetate was inhibited 85% by 10 μM NaCN (137 nmol min^{-1} mg of protein^{-1} to 20 nmol min^{-1} mg of protein^{-1}), while methanogenesis from methanol (55 nmol min^{-1} mg of protein^{-1}) was not affected by NaCN. The rate of methanogenesis from methanol or methylcoenzyme M in methanol-grown cell extracts was not affected by 10 mM NaCN (data not shown).

The effect of NaCN on the catabolism of mixtures of methanol and acetate was also investigated. In methanol-acetate or H_2-CO_2–acetate mixtures, part of the methyl group of acetate is oxidized to CO_2, and electrons generated from the oxidation are used to reduce methanol or CO_2 to CH_4 (8, 17, 28). NaCN (10 μM) had a strong inhibiting effect on CH_4 and CO_2 production from the methyl group of acetate but did not inhibit CH_4 or CO_2 production from methanol (Table 4).

NaCN caused a 50% increase in $^{14}CO_2$ production by acetate-grown cell suspensions incubated with [^{14}C]methanol and methanol plus acetate but had little effect on the amount of $^{14}CH_4$ produced. When sodium [2-^{14}C]acetate was substituted for [^{14}C]methanol, $^{14}CH_4$ production and $^{14}CO_2$ production were inhibited equally (59%). This effect was also evident in the ratio of $^{14}CH_4$ to $^{14}CO_2$ produced from the

TABLE 4. Influence of cyanide on conversion of acetate and methanol to CH_4 and CO_2 by resting cell suspensions of *M. barkeri*[a]

Labeled substrate	NaCN (μM)	dpm/μmol		
		$^{14}CH_4$	$^{14}CO_2$	$^{14}CH_4/^{14}CO_2$
$^{14}CH_3OH$	0	44,400	12,300	3.6
$^{14}CH_3OH$	10	40,600	18,000	2.3
[2-^{14}C]acetate	0	45,300	61,900	0.7
[2-^{14}C]acetate	10	18,500	27,100	0.7

[a] Cultures for preparation of cell suspensions were grown on complex medium with 50 mM sodium acetate. Methods for preparation of suspensions and incubation conditions are described in the text. Incubations were performed in duplicate or triplicate in PD buffer containing 89 μg of cell protein. Reaction mixtures received 10 mM methanol, 1 mM acetate, and 2 μCi of labeled substrate in an N_2 atmosphere.

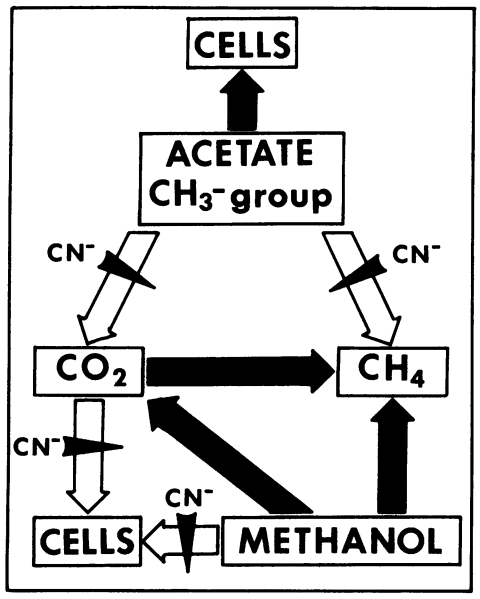

FIG. 1. Points at which cyanide inhibits metabolism of *M. barkeri*.

methanogenic substrates. This indicates that the oxidation of acetate but not methanol methyl groups to CO_2 was via a cyanide-sensitive pathway.

DISCUSSION

Our experiments agree with previous work showing that acetate catabolism to CH_4 and CO_2 in *M. barkeri* is inhibited by lower (10 μM) NaCN concentrations than those required to inhibit methanogenesis from H_2-CO_2 or methanol (7; Smith et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1983). We also found that methanogenesis from methylamine or trimethylamine was relatively insensitive to NaCN inhibition.

From the growth experiments, it is clear that NaCN exhibited two inhibitory effects: (i) inhibition of acetate catabolism and (ii) inhibition of CO_2 (as the sole carbon source) and of methanol assimilation. The first effect was manifest in complex medium; the second occurred in mineral or defined medium containing methanol or H_2-CO_2. Since the same effects occurred in mineral medium as in defined medium, we concluded that cysteine cannot replace acetate as a sole source of carbon or abolish inhibition by NaCN. The specific inhibition of acetate catabolism was confirmed by the experiments with cell suspensions and cell extracts. The inhibition of anabolism from CO_2 or methanol was confirmed by labeling experiments with radioactive substrates in which assimilation of methanol but not growth was inhibited. The points at which NaCN inhibited metabolism in *M. barkeri* are summarized in Fig. 1.

Inhibition by NaCN of acetate catabolism was not limited to methanogenesis. Acetate-grown cells metabolizing a mixture of methanol and acetate or H_2-CO_2 and acetate produce CH_4 simultaneously from methanol and acetate (8, 17, 21). In addition, part of the acetate methyl group metabolized is oxidized to CO_2, which provides electrons for increased CH_4 production from methanol (28). Our experiments showed that 10 μM NaCN inhibited equally methanogenesis from acetate and oxidation of the methyl carbon atom of acetate to CO_2. This suggests that the source of methyl groups from acetate for oxidation to CO_2 was the methanogenic aceticlastic reaction. Methyl-group oxidation in methanogenic bacteria has not been studied in any detail even though it has an essential role in catabolism.

Inhibition of carbon assimilation from methanol or CO_2 by 10 μM NaCN has not been reported for *Methanosarcina* spp. but was reported for CO_2 metabolism by *Methanobacterium thermoautotrophicum* (7). At the low acetate concentrations (2 mM) used in our experiments with complex medium, approximately 2 mol of carbon were assimilated from methanol for each mol of carbon assimilated from acetate. When 10 μM NaCN was added to the cultures, carbon from methanol and acetate was assimilated in equal proportions, and growth rates were not decreased. It is believed that methanol and CO_2 are assimilated into cell carbon by first being converted to acetate (or acetyl coenzyme A) (12), but this has been shown only for CO_2 (13). Our experiments show that when assimilation of CO_2 or methanol is blocked by NaCN, the cells are able to increase the rate of uptake and assimilation of exogenous acetate to maintain their growth rate. In a defined medium not containing exogenous acetate, the growth rates must slow down in the presence of NaCN because of carbon limitation.

It has been suggested that the synthesis of acetate from one-carbon units for anabolism is accomplished by the same enzyme system operating in reverse direction as the cleavage of acetate to CH_4 and CO_2 (15, 27). However, there is no evidence for this. CO dehydrogenase was proposed as a candidate for that enzyme because it is implicated in acetogenesis from CO_2 by acetogenic bacteria and is present in methanogens such *M. thermautotrophicum* that synthesize acetate from CO_2 for anabolic purposes (5, 9, 18). CO dehydrogenase occurs with high specific activity in extracts from acetate-grown cultures of *M. barkeri* in which acetate synthesis is not required (12). The purified enzyme is inhibited by 10 μM NaCN(15). Our results are consistent with the suggestion that CO dehydrogenase has a dual metabolic function. If the suggestion is correct, then CO dehydrogenase also functions in the oxidation of acetate methyl groups but not methanol methyl groups to CO_2. This could give rise to the preferential oxidation of acetate to CO_2 over other substrates observed with substrate mixtures and might explain the observation of Blaut and Gottschalk (3) that up to half of the acetate methyl group is oxidized to the level of a methylene group before being reduced to CH_4.

NaCN inhibition experiments with *M. thermoautotrophicum* indicated that CO dehydrogenase functioned in the synthesis of acetyl coenzyme A for carbon assimilation by reducing one molecule of CO_2 to the level of a carboxyl group and then attaching the carboxyl group to a methyl group that was derived from a second molecule of CO_2 (23–25). Synthesis of the methyl group of acetyl coenzyme A from CO_2 involved a separate pathway that was insensitive to cyanide. Our experiments are consistent with CO dehydrogenase involvement in assimilation of methanol in *M. barkeri*.

NaCN inhibition in some respects resembled inhibition reported for the corrinoid antagonist iodopropane (12), but with some clear differences. Both NaCN and iodopropane inhibition were prevented by adding acetate to the growth medium, an indication that both inhibitors blocked the synthesis of acetate from one-carbon units. However, iodopropane did not selectively inhibit assimilation of methanol in short- or long-term in vivo labeling experiments (12, 13). NaCN, however, did selectively inhibit methanol assimilation into cell carbon. This difference suggests that NaCN did not inhibit methanol assimilation as a corrinoid antago-

nist. Our experiments suggest that NaCN inhibited methanol assimilation by inhibiting CO dehydrogenase, because NaCN but not iodopropane inhibits purified CO dehydrogenase (15).

NaCN inhibition in *M. barkeri* differed from NaCN inhibition in *M. thermoautotrophicum* in not being prevented by acetate (23–25). This suggests that essential cyanide-sensitive pathways of carbon metabolism that exist in *M. thermoautotrophicum* are not operative in *M. barkeri*.

ACKNOWLEDGMENTS

We thank L. Baresi and R. A. Mah for valuable discussion and Mary C. Sullivan and M. Weiss for technical assistance.

This work was conducted at WRRC and supported in part by a grant from the Fulbright-Ministry of Education and Science of Spain Commission.

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