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Assimilatory Reduction of Sulfate and Sulfite by Methanogenic Bacteria

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A variety of sulfur-containing compounds were investigated for use as medium reductants and sulfur sources for growth of four methanogenic bacteria. Sulfide (1 to 2 mM) served all methanogens investigated well. *Methanococcus thermolithotrophicus* and *Methanobacterium thermoautotrophicum* Marburg and ΔH grew well with S^0, SO_3^{2-}, or thiosulfate as the sole sulfur source. Only *Methanococcus thermolithotrophicus* was able to grow with SO_4^{2-} as the sole sulfur source. 2-Mercaptoethanol at 20 mM was greatly inhibitory to growth of *Methanococcus thermolithotrophicus* on SO_4^{2-} or SO_3^{2-} and *Methanobacterium thermoautotrophicum* Marburg on SO_3^{2-} but not to growth of strain ΔH on SO_3^{2-}. Sulfite was metabolized during growth by *Methanococcus thermolithotrophicus*. Sulfide was produced in cultures of *Methanococcus thermolithotrophicus* growing on SO_4^{2-}, SO_3^{2-}, thiosulfate, and S^0. *Methanobacterium thermoautotrophicum* Marburg was successfully grown in a 10-liter fermentor with S^0, SO_3^{2-}, or thiosulfate as the sole sulfur source.

The methane-producing bacteria are a relatively unstudied group of bacteria that make up one of the larger divisions of the archaebacteria (1, 11, 12, 31). Most methanogens will use H_2 plus CO_2 as the major or sole source of carbon for energy generation and cell material. A variety of techniques and media for the growth of these strict anaerobes have been described previously (1, 2). Most notably, a recent technical advance with tubes or bottles pressurized with the substrate H_2-CO_2 (80:20, vol/vol) has facilitated small-scale growth of these bacteria; this system involves using O_2-free medium reduced with Na_2S or cysteine to assure strict anaerobic condition; titanium-citrate and titanium-nitrilotriacetate have also been used in medium reduction (18, 35). However, large-scale growth in fermentors routinely involves a bubbling gas system in which H_2S is slowly flushed from the medium, causing an ultimate slowing of growth unless sulfide is re-added. Further, the H_2S presents a great odor problem in the laboratory during both growth and harvesting.

Little is known of how methanogens obtain their sulfur for growth. The importance of sulfide to the growth of *Methanosarcina*, *Methanococcus*, and *Methanobacterium* species has been demonstrated (6, 8, 14, 17, 19, 22–24, 30, 32), and recently Stetter and Gaag (27) and Daniels et al. (10; L. Daniels, N. Belay, and D. Dingman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, I33, p. 127) have shown that many methanogens can reduce elemental sulfur to sulfide. Bhatnagar et al. (5, 6) have recently examined the use of several sulfur sources (in the presence of 2-mercaptoethanol [2-ME]) by two *Methanobacterium* species. There has been little information published describing the variety of inorganic sources of sulfur that methanogens can use for growth (31). We report here the effects of a wide range of sulfur-containing compounds (especially sulfate, sulfite, and thiosulfate) on the growth of four methanogens, *Methanococcus thermolithotrophicus*, *Methanobacterium thermoautotrophicum* ΔH and Marburg, and *Methanospirillum hungatei*. We also describe how some previously unknown sulfur sources provide for the large-scale growth of methanogens.

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MATERIALS AND METHODS

Organisms. *Methanococcus thermolithotrophicus* was obtained from K. O. Stetter (16), and *Methanobacterium thermoautotrophicum* ΔH (DSM 1053 = ATCC 29096) was a gift of J. Winter (36). *Methanobacterium thermoautotrophicum* Marburg (DSM 2133) was provided by Georg Fuchs (7, 13). *Methanospirillum hungatei* GPI was a gift of G. D. Sprott (21).

Medium and growth conditions. Cells were grown by a modification of the method of Balch and Wolfe (2). *Methanococcus thermolithotrophicus* was grown at 62°C in medium resembling seawater, modified from that previously described (16); it consisted of the following components (millimolar) in distilled and deionized water: KCl (4.1), NH_4Cl (16.8), $CaCl_2 \cdot 2H_2O$ (0.61), K_2HPO_4 (1.45), KH_2PO_4 (1.85), NaCl (385), $MgCl_2 \cdot 6H_2O$ (38), Na_2CO_3 (1.5), resazurin (0.003). $MgCl_2$ was added separately after autoclaving. A 100-fold-concentrated solution of trace elements was added to this medium to give the following final concentrations (micromolar): nitrilotriacetic acid (71), $MnCl_2 \cdot 4H_2O$ (4.5), $FeCl_2 \cdot 4H_2O$ (6.8), $CaCl_2$ (4.1), $CoCl_2 \cdot 6H_2O$ (7.6), $ZnCl_2$ (6.6), $CuSO_4$ (2.8), $Na_2MoO_4 \cdot 2H_2O$ (1.9), $NiCl_2 \cdot 6H_2O$ (3.8). The final pH was about 6.2.

Methanobacterium thermoautotrophicum Marburg and ΔH were grown at 62°C in medium that consisted of the following components (millimolar): KH_2PO_4 (3.09), K_2HPO_4 (1.26), $MgCl_2 \cdot 6H_2O$ (0.19), $CaCl_2 \cdot 2H_2O$ (0.21), NH_4Cl (13.08), NaCl (10.27), resazurin (0.003), Na_2CO_3 (1.51). A 100-fold- concentrated solution of trace elements was added to this medium to give the following final concentrations (micromolar): nitrilotriacetic acid (78.4), $FeCl_2 \cdot 6H_2O$ (20.7), $CoCl_2 \cdot 6H_2O$ (0.21), $Na_2MoO_4 \cdot 2H_2O$ (0.20), $NiCl_2 \cdot 6H_2O$ (4.2). The pH was adjusted to 7 by the addition of Na_2CO_3 while bubbling the medium with H_2-CO_2 (80:20, vol/vol).

Methanospirillum hungatei was grown at 37°C in medium that contained the following components (millimolar): K_2HPO_4 (4.7), $MgCl_2 \cdot 6H_2O$ (0.3), NH_4Cl (7.5), $CaCl_2 \cdot 2H_2O$ (0.44), NaCl (5.1), sodium acetate (20); the mineral elixir and resazurin were as described above for *Methanococcus thermolithotrophicus*; 10 ml of vitamin mix

(33) per liter was added. The pH was adjusted to 7 in the same manner as for strain ΔH.

After preparation, all types of media were dispensed into aluminum seal tubes (no. 2048-00150; Bellco Glass, Inc., Vineland, N.J.) in 5-ml amounts. The tubes were sealed with lipped black stoppers (aluminum seal rubber stoppers, no. 2048-11800; Bellco), crimped with aluminum seals (no. 224183; Wheaton Scientific), and made anaerobic by repeated thorough evacuation and flushing with H_2-CO_2 (80:20, vol/vol), using thick red rubber hoses fitted with cut-off glass-metal Luer-lock 3-ml syringes with glass wool inside and a 25-gauge needle. Elemental sulfur was added as a solid before making the media anaerobic. When sulfide was the sulfur source, $Na_2S \cdot 9H_2O$ (1.8 mM, final concentration) was added by syringe from an anaerobic stock solution after the media were made anaerobic. The tubes, with about 70 kPa overpressure of H_2-CO_2, were then autoclaved. Breakage of the tubes during this procedure owing to vacuum or pressure is very rare. After sterilization, sodium salts of sulfate, sulfite, or thiosulfate were added to the tubes containing medium without sulfide or S^0 by sterile syringe from sterile stock solutions (sterilized by either filtration or autoclaving) prepared under a gas phase of argon. Filter-sterilized anaerobic 2-ME was similarly added at this time. Before inoculation, the tubes (except those containing sulfide as a sulfur source, which were pressurized after inoculation) were re-evacuated and pressurized with 140 kPa H_2-CO_2 (80:20, vol/vol), using sterile hoses.

Inocula for sulfur source experiments were prepared in 250-ml stoppered bottles (no. 223950; [Wheaton Scientific] sealed with cut-off no. 1 black stoppers and 30-mm [outer diameter] aluminum seals [no. 224187; Wheaton]) containing 50 ml of medium and made anaerobic as described above; owing to the larger volume, evacuation and gassing require considerably more time than with tubes. (Unlike the safety record of tubes, we have had two hot-bottle explosions in our lab. We have developed a protective cover from a large plastic bottle for autoclaving [9].) The inoculum media contained 0.8 mM sulfide, thus giving a maximum of about 80 μM carry-over sulfide in the inoculum for the experiments, since inocula were typically 10%. Note that in our discussion of sulfide concentrations, at the pH of growth more than half the total sulfide is in the gas phase, and thus these mentioned background or control levels are higher than are actually present in the experimental tubes.

Incubation was carried out with the tubes horizontal in a gyratory shaker at 150 rpm unless indicated otherwise. Growth was measured by the A_{600} with a Spectronic 20 spectrophotometer. All datum points were averages of triplicate tubes.

Fermentor growth of the *Methanobacterium* species on S^0, thiosulfate, and SO_3^{2-} was conducted as described elsewhere (10).

All the cultures used in this study were examined for heterotrophic contaminants by microscopic examination (phase-contrast); by inoculation into medium under N_2-CO_2 (80:20, vol/vol) atmosphere supplemented with 0.2% each yeast extract and peptone and 0.05% each glucose, sucrose, xylose, and glycerol; and by growth on H_2-CO_2 with sulfide or other sulfur sources (SO_4^{2-}, SO_3^{2-}, or thiosulfate depending on growth in these substrates) in the presence of antibiotics (52 to 78 μg each of streptomycin and kanamycin per ml for *Methanococcus thermolithotrophicus*, 76 μg of streptomycin per ml for *Methanobacterium thermoautotrophicum* Marburg and ΔH, and 52 μg each of streptomycin and vancomycin per ml for *Methanospirillum hungatei*) known to affect eubacteria but not methanogens. *Methanococcus thermolithotrophicus* was also examined for growth in medium supplemented with 10 mM lactate and sulfate, 0.02% yeast extract, and vitamin mix (used for SO_4^{2-} reducers). None of the cultures showed growth in the heterotrophic medium and grew well in the presence of antibiotics. *Methanococcus thermolithotrophicus* was plated out in an anaerobic glove bag on Gelrite (Kelco, San Diego, Calif.) plates, using previously described techniques (2, 17). Agar proved to be a poor agent for petri plate growth.

Analysis of sulfur-containing compounds. The gas phase (by direct injection [3]) and the liquid phase (by benzene extraction [29]) of uninoculated and inoculated tubes containing different sulfur compounds were analyzed for volatile sulfur compounds with a Shimadzu GC 9A chromatograph equipped with a flame photometric detector (394 nm) and a Teflon (fluorinated ethylene propylene) column (3 m by 3.2 mm [inner diameter]) packed with Chromosil 330. The gas chromatograph was operated at 70°C with an H_2 flow rate of 105 ml/min, an He flow rate of 50 ml/min, and an air flow rate of 55 ml/min. Also, dissolved sulfide was measured spectrophotometrically by the methylene blue assay (28). For determination of sulfide from SO_4^{2-}, SO_3^{2-}, S^0, or thiosulfate, *Methanococcus thermolithotrophicus* was grown in tubes sealed with 20-mm gold-top seals with tetrafluoroethylene-faced butyl rubber liners (no. 6689; Alltech Associates, Inc., Deerfield, Ill.). The levels of SO_3^{2-} in both inoculated and uninoculated tubes during different periods of incubation were quantitated spectrophotometrically as described previously (28).

RESULTS

Observations during medium preparation and inoculation. Resazurin is a redox dye included during preparation of media for methanogens. Initially, the oxidized dye appears purple in the medium; slight reduction yields a pink color; complete reduction clears the medium. When no reducing agent is added to tubes or bottles, even when the systems are fully oxygen-free, the purple color remains. Thus resazurin is a useful indicator of: the effectiveness of a compound as a reducing agent; the production of reduced sulfur compounds; and the redox state of an inoculated culture, especially important for seeing whether conditions are reduced enough to support growth. We observe that autoclaving under an N_2 or argon gas phase with medium containing 1 to 2 mM sulfide leads to full reduction; 1 to 2 mM elemental sulfur and sulfite result in partial reduction; and sulfate and thiosulfate do not reduce the system at all. In most of the experiments described below, sulfur-containing compounds (except 2-ME) were autoclaved separately under an argon gas phase. 2-ME (filter sterilized) cleared the medium at a $\geq$2.5 mM concentration. Culture media prepared for control experiments contained no added sulfide or other sulfur source, but cleared readily upon 5 to 10% inoculation from 0.7 mM sulfide-grown cultures.

Sulfur sources used for growth. Table 1 describes the abilities of several sulfur compounds to serve as sulfur sources for growth of methanogens. The data describe experiments done in either the presence or absence of 2-ME. In all cases, carry-over sulfide (0.07 mM) or 2-ME (2.5 to 20 mM) did not result in growth, while added sulfide (1.8 mM) allowed full growth. While the initial growth on nonsulfide sulfur sources was sometimes inconsistent in tubes fully agitated immediately after inoculation, standing incubation of the tubes for 12 to 20 h after inoculation resulted in fully reproducible results; after the initial adaptation to these

TABLE 1. Use of various sulfur sources for growth by methanogens

Sulfur source	Substrate concn (mM)	Maximum A^{600a}							
		Methanococcus thermolithotrophicus		*Methanobacterium thermoautotrophicum*				*Methanospirillum hungatei*	
				Marburg		ΔH			
		A	B	A	B	A	B	A	B
Sulfide[b]	0.07	0.03	0.03	0.05	0.03	0.06	0.06	0.07	0.04
Sulfide	1.90	0.80	0.84	0.90	0.50	1.05	1.00	0.95	0.80
Sulfate	4.4	0.75	0.08	0.09	0.15	0.08	0.09	0.15	0.10
Thiosulfate	4.0	0.75	0.80	0.80	0.85	0.45	0.85	0.09	0.43
Sulfite	0.50	0.75	0.04	0.85	0.08	0.90	0.95	0.12	0.06
Sulfur	3.6 mg/ml	0.70	0.86	0.90	1.05	0.95	1.10	0.09	0.85

[a] Maximum A_{600} was recorded after incubation for 15 to 20 h for *Methanococcus thermolithotrophicus*, 20 to 90 h for *Methanobacterium thermoautotrophicum* Marburg, 20 to 120 h for strain ΔH, and 4 to 12 days for *Methanospirillum hungatei*. An A_{600} of less than 0.2 is taken as no growth. A, Sulfur source without 2-ME; B, sulfur source with 20 mM 2-ME for *Methanococcus thermolithotrophicus* and *Methanobacterium thermoautotrophicum* Marburg and ΔH and 2.5 mM for *Methanospirillum hungatei*.

[b] Residual from inoculum.

sulfur sources, cultures were transferred repeatedly without a lag. All data in Table 1 refer to tubes incubated initially under these nonshaking conditions, but then grown while shaking. *Methanococcus thermolithotrophicus* gave identical results (as in Table 1) when filter-sterilized sulfur sources (except S^0) were used. Also, we have observed with *Methanococcus thermolithotrophicus* that the A_{600} parallels methane production during growth on all sulfur sources (data not shown).

(i) Sulfide. In an initial experiment the effect of sulfide levels (0.1 to 10 mM) on the growth of *Methanococcus thermolithotrophicus*, *Methanobacterium thermoautotrophicum* Marburg and ΔH, and *Methanospirillum hungatei* was studied. Most of these organisms preferred sulfide levels of 1 to 2 mM for optimum growth; *Methanococcus thermolithotrophicus* was more sensitive to higher sulfide levels, being inhibited by >2 mM (data not shown). Growth of *Methanospirillum hungatei* on sulfide was inhibited at >5 mM 2-ME (Fig. 1), while growth of *Methanobacterium thermoautotrophicum* Marburg was inhibited at 20 mM (Table 1). Growth of *Methanococcus thermolithotrophicus* and *Methanobacterium thermoautotrophicum* ΔH on sulfide was not inhibited even at 20 mM 2-ME (Table 1).

(ii) Sulfate. None of the organisms except *Methanococcus thermolithotrophicus* were able to grow on sulfate as a sulfur source, either in the presence or in the absence of 2-ME (2.5 to 5 mM). The ability of *Methanococcus thermolithotrophicus* to use sulfate was confirmed by repeated transfer. However, when 2-ME was lacking, oxidation of the medium and failure of growth were often problems in repeated transfers unless the cultures were allowed to stand at the incubation temperature for 8 to 15 h before shaking was initiated. The level of SO_4^{2-} required for optimal growth was 1 to 2 mM, and higher concentrations had no further effect

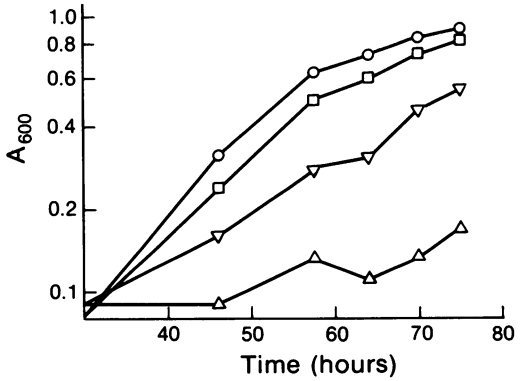

FIG. 1. Inhibition of growth by 2-ME of *Methanospirillum hungatei* in medium containing sulfide (1.9 mM) as the sulfur source. Symbols: ○, no 2-ME added; □, 4.9 mM 2-ME; ▽, 12.2 mM 2-ME; △, 24.5 mM 2-ME.

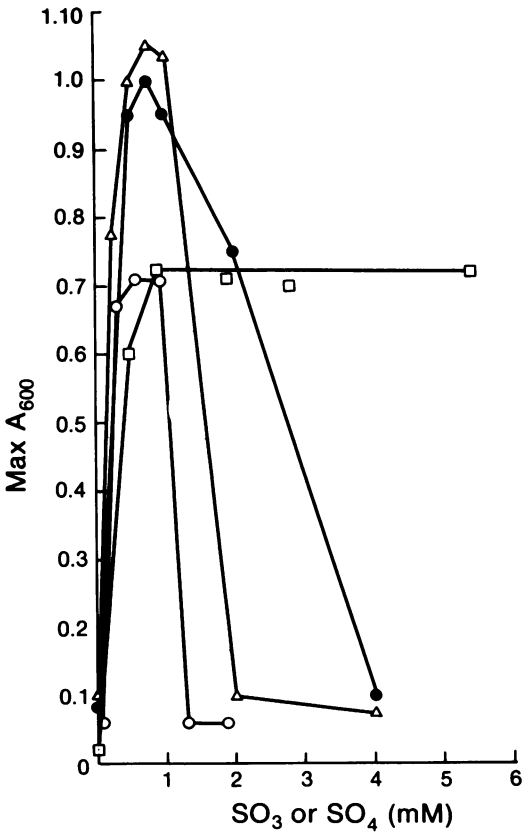

FIG. 2. Effects of various concentrations of sulfate (□) and sulfite (○) on growth of *Methanococcus thermolithotrophicus* and sulfite concentrations on growth of *Methanobacterium thermoautotrophicum* ΔH (●) and Marburg (△).

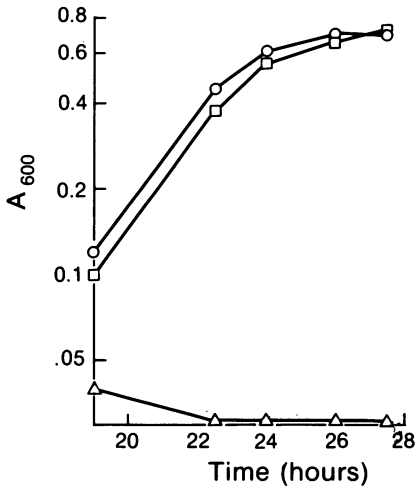

FIG. 3. Inhibition of growth by 2-ME of *Methanococcus thermolithotrophicus* in medium containing sulfite as the sulfur source. Cultures were incubated without shaking for 19 h. The growth medium contained 0.53 mM sodium sulfite as a sulfur source. Symbols: ○, no 2-ME added; □, 5 mM 2-ME; △, 20 mM 2-ME.

(Fig. 2). Growth on SO_4^{2-} as the sulfur source was inhibited by 20 mM 2-ME (Table 1).

(iii) Sulfite. *Methanococcus thermolithotrophicus* and *Methanobacterium thermoautotrophicum* Marburg and ΔH were able to grow on SO_3^{2-} as the sulfur source in the presence or absence of 2-ME (2.5 to 5 mM). 2-ME at 20 mM was inhibitory to the growth of *Methanococcus thermolithotrophicus* (Fig. 3) and *Methanobacterium thermoautotrophicum* Marburg but not to the growth of strain ΔH. After four repeated transfers, the effect of SO_3^{2-} concentration on growth was studied; optimal growth of all three organisms occurred at 0.5 to 1.0 mM, and 2.0 mM was greatly inhibitory to growth of *Methanococcus thermolithotrophicus* and *Methanobacterium thermoautotrophicum* Marburg (Fig. 2).

(iv) Thiosulfate. *Methanococcus thermolithotrophicus* and *Methanobacterium thermoautotrophicum* Marburg were able to grow well on thiosulfate as a sulfur source in the absence of 2-ME; strain ΔH showed little growth (Table 1). However, when 2-ME (2.5 to 5 mM) was included, growth of strain ΔH with thiosulfate was greatly improved, and *Methanospirillum hungatei* also showed considerable growth under these conditions (Table 1). As discussed later, the improved growth on thiosulfate in the presence of 2-ME is probably due to the result of more reduced forms of sulfur produced by interactions between thiosulfate and 2-ME. Growth of *Methanococcus thermolithotrophicus* and *Methanobacterium thermoautotrophicum* Marburg and ΔH on thiosulfate as a sulfur source was not inhibited by 20 mM 2-ME (Table 1). After four transfers, the effect of thiosulfate concentration on growth was studied; optimal growth occurred at 1 to 2 mM with *Methanococcus thermolithotrophicus* and at 3 to 4 mM with strain Marburg, and higher concentrations had no further effect (data not shown).

(v) Elemental sulfur. All the organisms except *Methanospirillum hungatei* were able to grow readily on elemental sulfur. None of the cultures showed any lag, and initial growth occurred under normal shaking conditions. Growth of *Methanococcus thermolithotrophicus* and both *Methanobacterium thermoautotrophicum* strains with elemental sulfur was not inhibited by 20 mM 2-ME (Table 1). Interestingly, growth of *Methanospirillum hungatei* with S^0 occurred when 2.5 to 5 mM 2-ME was included in the medium; as discussed later, it is likely that the growth is due to production of more reduced forms of sulfur resulting from interaction between elemental sulfur and 2-ME.

Sulfur source for fermentor-grown cells. Several consecutively inoculated fermentors of *Methanobacterium thermoautotrophicum* Marburg (Microgen; New Brunswick Scientific Co., Inc., Edison, N.J.) gassed with H_2-CO_2 were successfully grown with elemental sulfur as the sole sulfur source. This resulted in a slow entry of reduced sulfur into the medium and an eventual conversion of the added S^0. The cells grew to high densities (approximately 1.0 g (dry weight) per liter, roughly equal to that of sulfide-grown cultures) and were maintained in a very reduced environment. With thiosulfate (10 mM), fermentor growth of approximately 1.0 g (dry weight) per liter was obtained. With sulfite (0.75 mM originally and added again to 0.75 mM at 35 h), fermentor growth of approximately 1.5 g/liter was achieved.

Examination of uninoculated and inoculated media for production of reduced sulfur compounds. The headspace and liquid of media containing different sulfur compounds were examined by gas chromatography to detect the abiological production of volatile sulfur compounds during incubation. Also, dissolved sulfide was measured by a chemical assay. The chemical assay was sensitive enough to detect only 40 to 300 μM sulfide, while the gas chromatographic method was much more sensitive (2 to 20 nM). Thiosulfate, sulfate, sulfite, and S^0 yielded no detectable volatile or benzene-extractable compounds during abiological incubation for 30 days, but when 2-ME (2.5 mM) was present with thiosulfate

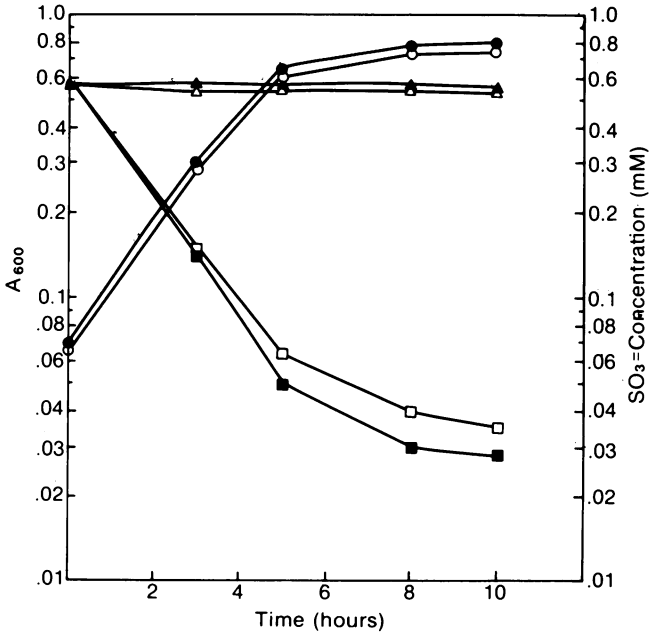

FIG. 4. Levels of sulfite (autoclaved or filter sterilized) in uninoculated tubes and tubes inoculated with *Methanococcus thermolithotrophicus* during incubation. Symbols: □, SO_3^{2-} (autoclaved) in inoculated medium; ■, SO_3^{2-} (filter sterilized) in inoculated medium; △, SO_3^{2-} (autoclaved) in uninoculated medium; ▲, SO_3^{2-} (filter sterilized) in uninoculated medium; ○, growth with 0.56 mM autoclaved SO_3^{2-}; ●, growth with 0.56 mM filter-sterilized SO_3^{2-}.

(4 mM) or S^0 (4 mg/ml), sulfide was produced at a level of 0.5 to 0.9 mM. The use of sulfite and its potential to produce other sulfur-containing compounds during incubation was examined by a chemical sulfite assay. The levels of sulfite (filter sterilized or autoclaved) in uninoculated tubes remained the same as the initial concentration (Fig. 4), while the levels in tubes inoculated with *Methanococcus thermolithotrophicus* decreased gradually with growth, demonstrating SO_3^{2-} metabolism by the growing cells. Growing cells of *Methanococcus thermolithotrophicus* produced sulfide from other S sources in various amounts. At the early stationary phase, the following sulfide levels were observed: SO_4^{2-}, 0.1 to 0.2 mM; SO_3^{2-}, 0.25 to 0.3 mM; S^0, 0.5 to 1.0 mM; thiosulfate, 0.4 to 0.6 mM.

DISCUSSION

Virtually all media previously described for the growth of methanogens contain sulfide added to make an initial concentration of 1 to 2 mM; cysteine (1 to 2 mM) has also been used in conjunction with sulfide (1, 2, 7, 8, 13, 14, 16, 17, 19, 21–24, 30, 32, 33, 36). However, few other potential sources of sulfur for medium reduction or growth requirements have been studied; indeed, some workers have concluded that fully reduced sulfur is required by all methanogenic bacteria (14, 23, 24, 31).

It is important to note that the sulfide concentrations given here and in other papers refer to initial sulfide concentrations. Since the pK for the $H_2S \rightleftarrows HS^-$ equilibrium is about 7, we can make the assumption that at pH 6, nearly all sulfide is present as H_2S. With knowledge of the gas volume (22 ml), the liquid volume (5 ml), and the α (1.19 volumes of gas dissolved in the liquid per volume of liquid at 60°C [15]), a sulfide concentration can be calculated. At pH 6 and 7, respectively, the equilibrium levels in the liquid are about 0.2 and 0.6 of the original concentration.

Previous work showed that *Methanosarcina barkeri* grew well with sulfide and could not grow with sulfate or cysteine alone (19, 24). *Methanococcus voltae* was reported to require sulfide for growth; cysteine, dithiothreitol, or sulfate did not suffice (32). Both *Methanobacterium thermoautotrophicum* ΔH and *Methanobacterium* strain *ivanovi* can use cysteine alone as a sulfur source (6). Bryant et al. (8) showed that *Methanobrevibacter ruminantium* and *Methanobacterium bryantii* did not use sulfate. A strain of *Methanobacterium thermoautotrophicum* examined by Rönnow and Gunnarsson (23) did not use cysteine, thiosulfate, or sulfate. *Methanosarcina barkeri* contains a sulfite reductase, although growth on sulfite has not been demonstrated (20). However, our work shows that a variety of sulfur-containing inorganic compounds, with the sulfur at several different oxidation states, can serve as the sole sources of sulfur for growth. Even the most oxidized sulfur compounds can serve as a sulfur source for some methanogens.

When testing methanogens for their ability to grow on sulfur sources other than sulfide, growth may be poor or may not occur at all when cultures are shaken as usual from the start of the incubation period. As indicated from the results given above, this phenomenon is particularly true when SO_3^{2-} is tested. In our preliminary work (10), the use of SO_3^{2-} by *Methanobacterium thermoautotrophicum* Marburg and ΔH and the use of thiosulfate by strain Marburg was not observed apparently because the standing incubation technique was not used. Cultures may be subjected to more oxidation under shaking conditions until they adapt to the new substrate. Therefore, we recommend that when growing methanogens on sulfur sources other than sulfide, a period of standing incubation should be allowed.

Caution should be used when sulfur-containing compounds are autoclaved or the incubation temperature is >80°C, since as reported by Belkin et al. (4), S^0 can disproportionate to form sulfide. Significant nonbiological dismutation might be expected, for example, when the medium pH is 7.5 or above and the incubation temperature is near 100°C; in 1 day, a 1 mM sulfide level might occur. Using their data on pH, temperature, and time, the autoclaving of our tubes containing S^0 should have produced sulfide at less than a 10 μM concentration, consistent with our observation of the slight resazurin reduction upon autoclaving (the resazurin in tubes turns pink at about 10 μM sulfide and clear at 70 to 100 μM; this is much lower than the approximate 300 μM needed to see significant growth). A similar phenomenon may occur with SO_3^{2-}, but the degree of medium reduction in our experiments suggests that the expected dismutation products are at about the same concentration as with S^0. Gas chromatographic evidence demonstrated that chemical-mediated sulfide production is negligible during autoclaving of a SO_3^{2-} solution or during incubation in the media. Furthermore, sulfite assays of tubes containing SO_3^{2-} (autoclaved or filter sterilized) demonstrated no significant loss of SO_3^{2-} in uninoculated tubes during incubation; but in tubes inoculated with *Methanococcus thermolithotrophicus*, SO_3^{2-} levels dropped significantly during growth, indicating its use (Fig. 4).

The growth of methanogens in medium containing thiosulfate at 4 to 6 mM concentrations should be viewed with caution. Both strains of *Methanobacterium thermoautotrophicum* grew best at levels above 3 mM; dismutation products of the thiosulfate may be acting as reductants and may be assimilated for growth. But examination of thiosulfate as well as S^0, SO_4^{2-}, or SO_3^{2-} medium by gas chromatographic and chemical methods showed no abiological production of sulfide or other sulfur compounds during incubation. *Methanococcus thermolithotrophicus* was also successfully grown at 37°C with all the sulfur sources, proving growth was not as a result of abiological reduction of oxidized sulfur sources owing to high-temperature incubation. We conclude that thiosulfate works as a sulfur source in the medium, but we are not certain whether it enters the cell directly or via a chemical dismutation product, although we can find no evidence for such a product. However, when 2-ME is included in the medium along with thiosulfate or S^0, chemical-mediated sulfide production occurs and makes interpretation difficult; it is likely that chemically produced sulfide is providing the sulfur source in these cases.

The work by Bhatnagar et al. (5) demonstrated that 2-ME does not serve as a sole sulfur source in the two strains of *Methanobacterium* they examined. This observation agrees with our results; however, we note that even though growth of *Methanococcus thermolithotrophicus* and *Methanobacterium thermoautotrophicum* ΔH with sulfide are not affected by 20 mM 2-ME, *Methanospirillum hungatei* and *Methanobacterium thermoautotrophicum* Marburg are inhibited by 10 to 20 mM. Further, the use of 2-ME may lead to a false conclusion that a sulfur compound is not used by a methanogen when in fact its utilization may be inhibited by 2-ME; indeed, we show here that growth on SO_3^{2-} or SO_4^{2-} is inhibited by this compound in some strains.

In examining potential sulfur sources, we feel that our strategy of using very low sulfide levels is preferred over the use of a higher level of nominally unmetabolized but potentially toxic reductant; the sulfide concentrations of 0.05 to

0.10 mM are not sufficient sulfur for growth but will reduce the medium to at least clear the resazurin upon inoculation, at which time the cells can start reducing the medium with the metabolizable sulfur source present. If 2-ME must be used, its effect on growth with sulfide or other sulfur sources should be carefully determined.

The sulfide and sulfite concentration curves suggest that it is very important for optimal growth to determine for each organism the optimal levels (as shown here and by other workers [17, 19, 22, 24, 32]). Bhatnagar et al. (5) used 5 mM SO_3^{2-} in testing methanogens for their growth on this compound; however, we found that more than 1 to 2 mM is greatly inhibitory to all strains we have tested so far. Care should be taken with elemental sulfur, since it could be metabolized rapidly to form toxic levels of H_2S.

Of practical interest, sulfide is not required by most of the methanogens examined. Thus, in the large-scale growth of these bacteria, the volatile and maldorous sulfide can be replaced by elemental sulfur, sulfate, sulfite, or thiosulfate, when appropriate. The conversion of sulfur to sulfide (first reported by Stetter and Gaag [27]) is indicated by the distinctive odor of the cultures and has been verified gas chromatographically (B. S. Rajagopal, unpublished data). Other workers have demonstrated the need of some method of maintaining sufficient sulfide levels in the medium during growth, since gassing the fermentor vessels with H_2-CO_2 leads to rapid depletion of total sulfide; at pH 6.2 (the normal pH under an H_2-CO_2 atmosphere), the predominant form of total sulfide is H_2S, a gas which goes out the fermentor exit. Most workers routinely add Na_2S solutions at intervals (several hours to about a day) (34; L. Daniels, unpublished observations); some have devised a method of providing H_2S as a gas in the incoming H_2-CO_2 mix (25, 26), yielding a constant sulfide level. The presence of a reservoir of a nonvolatile sulfur source makes the supplementation unnecessary, improving the ease of fermentor growth and reducing odor problems during growth and harvesting. However, excess particulate S^0 can present a problem during cell recovery.

The reduction of the sulfur-containing compounds with hydrogen as the reductant is thermodynamically favorable: SO_4^{2-}, SO_3^{2-}, and S^0 reduction to S^{2-} yields free energies ($\Delta G^{0'}$) of -152, -173, and -28 kJ per reaction, respectively. Several anaerobic archaebacteria, including *Thermoproteus*, *Desulfurococcus*, and *Thermococcus* species, obtain all their energy from S^0 reduction (37–39); *Desulfovibrio* species obtain their energy from SO_4^{2-} reduction. However, in many bacteria, assimilatory sulfate reduction supplies cells with a sulfur source, but no energy. It is likely that in methanogens the assimilatory route occurs, but the possibility exists that they can produce ATP by H_2S production; the distinction between these two possibilities is a relevant topic for future investigation.

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