Effect of Sulfur-Containing Compounds on Anaerobic Degradation of Cellulose to Methane by Mixed Cultures Obtained from Sewage Sludge†

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Tests were made to determine the effects of inorganic and organic sulfur sources on the degradation of cellulose to methane in a chemically defined medium with sulfur-poor inoculum prepared from sewage sludge. The results show that a sulfur source of about a 0.85 mM concentration is essential for the degradation of cellulose to CH₄. However, the production of CH₄ from CO₂ and H₂ provided in the headspace occurred with 0.1 mM sulfate or sulfide. At a 9 mM concentration, all inorganic sulfur compounds other than sulfite inhibited both cellulose degradation and methane formation, and this inhibition increased in the order thiosulfate < sulfite < sulfide < H₂S. It appears that the degradation of cellulose to CH₄ in a sulfate-free medium by inoculum maintained in a low-sulfur medium is inhibited because of the lack of availability of sulfur for growth of bacteria and synthesis of cell materials and sulfur-containing cofactors involved in cellulose degradation and methanogenesis. The reduction of methanogenesis by higher levels of sulfate probably occurs as a result of stimulation of reactions converting acetate and H₂ to end products other than CH₄.

Cellulose, one of the Earth's most abundant biopolymers, is increasingly recognized as an important potential energy source. One method for converting and upgrading the energy from cellulose is by producing methane through anaerobic digestion. Although methane formation from sewage sludge, which contains a substantial amount of cellulose, has been known for a long time, the fundamental biochemistry and microbiology of the process and the role played by various elements in the process are still poorly understood (28). For example, the literature on anaerobic fermentation indicates the necessity, as well as the undesirability, of the presence of sulfate and sulfides for degradation of solids to methane. In the anaerobic digestion of waste, the presence of sulfides has been shown to precipitate toxic heavy metals such as chromium, cobalt, copper, iron, nickel, and zinc (14, 15). In rumen microorganisms, inorganic sulfur has been implicated in the synthesis of sulfur-containing organic compounds which are incorporated into microbial proteins (9, 10). For the cultivation of anaerobes, sulfur-containing compounds have been employed to maintain a reducing environment essential for the growth of these organisms (6), and they form an integral part in ferredoxin (29) and other compounds involved in electron transport systems (20), as well as coenzyme M, which is involved in the methyl group transfer reactions in methanogenesis (16, 26). On the other hand, sulfides have been shown to be toxic to many anaerobes at fairly low concentrations (17, 34) and sulfates have been shown to inhibit methanogenesis (7, 19, 34). This multiple role of inorganic sulfur necessitated work to determine the effect of various sulfur-containing compounds on cellulose degradation and methane formation by mixed cultures. This paper reports the effects of a variety of sulfur-containing compounds on cellulose degradation to methane and the requirements for sulfur-containing compounds to optimize this process.

MATERIALS AND METHODS

Preparation of inoculum and medium. The effluents from a municipal sludge digester were diluted with an equal volume of medium and grown in a 10-liter fermentor (Sovirel, Lavallois-Perret, France) held at 35°C under anaerobic conditions. This fermentor was fed once a day and maintained using a sulfur-free chemically defined medium under the "repeated-fed-batch culture" technique (25) for 8 to 10 weeks. The medium contained (mg/liter): tissue paper pulp (5, 000), NH₄HCO₃ (3,000), KH₂PO₄ (450), KH₂PO₄ (450), Na₂CO₃ (320), NH₄Cl (180), MgCl₂·6H₂O (165), CaCl₂·2H₂O (120), NaCl (55), FeCl₃ (12), MnCl₂·4H₂O (5), CoCl₂·6H₂O (1), ZnCl₂ (0.8), CuCl₂·2H₂O (0.1), H₃BO₃ (0.1), Na₂MoO₄·2H₂O (0.1), pyridoxine HCl (0.1), thiamine HCl (0.06), riboflavin (0.05), nicotinic acid (0.06), biotin (0.02), folic acid (0.02), and vitamin
B₂ (0.005). The composition of this salt-vitamin mixture was adapted from the work of Bryant and Robinson (5), Holdeman and Moore (13), and Wolin et al. (36) by replacing all the cations present as sulfate with their respective chlorides on an equimolar basis. The feeding rate and retention time were 1.0 g of cellulose per liter per week and 16 to 20 weeks, respectively. This incubation procedure was followed principally to reduce the concentration of sulfur-containing materials present in the inoculum obtained from the sludge digester.

Further removal of sulfur-containing compounds from this inoculum was carried out by removing the supernatant and washing the cells. Portions of the fermenter liquid from the 10-liter fermentor were passed through six layers of cheese cloth to remove undegraded cellulose and transferred to 160-ml serum vials under anaerobic conditions (50 ml/vial). The vials were sealed, and the headspace in each vial was flushed and pressurized to 20 lb/in² with a 20% CO₂−80% H₂ mixture before centrifuging at 650 × g for 30 min. Higher speeds were not used because of the inability of the vials to withstand any greater centrifugal forces and the lack of centrifuge equipment for strict anaerobic work. However, the inoculum thus obtained metabolized cellulose to CH₄ effectively and was found suitable for comparative studies (Table 1). The supernatant from each vial was removed with a hypodermic syringe and was replaced, on a volumetric basis, by the sulfate-free salt-vitamin mixture. The cells were resuspended in this solution and used as an inoculum without any dilution. All preparations were carried out under an 80% H₂−20% CO₂ mixture at 35°C.

Culture conditions. Experiments were carried out in 160-ml serum vials (22) containing 250 mg of homogenized tissue paper in 50 ml of sulfur-free salt-vitamin mixture. This medium was supplemented with the desired amount of sulfur by anaerobically injecting aqueous solutions of Na₂SO₃, Na₂SO₄, Na₂S, H₂S, or a mixture of cysteine and methionine, which was prepared and boiled under the H₂−CO₂ mixture. This step was necessary to prevent the oxidation of some of these compounds, especially Na₂S. To test the effects of low oxidation-reduction potentials on cellulose breakdown to CH₄, limited tests were also conducted by adding titanium-citrate buffer (37), in 80-ml/liter concentrations, as a reducing agent. The headspace of all the vials was flushed with 80% H₂−20% CO₂ mixture. This gas mixture was selected for two reasons: (i) to maintain an anaerobic atmosphere and (ii) to determine the effects of these sulfur-containing compounds on methanogenesis occurring as a result of CO₂ reduction. The rate and extent of CH₄ formation from headspace gases also provided information on any possible batch-to-batch variation in the activity of methanogens present in the inoculum. For the calculation of results on a cellulose degradation basis, the volume and composition of the gas were corrected for conversion of headspace gas to CH₄ by using stoichiometry 4H₂ + CO₂ → CH₄ + 2H₂O. Since in mixed cultures the molar ratio of H₂ conversion to CH₄ formed range from 5:1 to 10:1 (33) rather than the theoretical ratio of 4:1, the amount of CH₄ formed from cellulose breakdown might be higher than calculated, and values for CH₄ formed from headspace gas might be correspondingly lower. All vials were incubated at 35°C with shaking. Results are averages for at least three tests carried out on different occasions. All tests were run in duplicate.

The redox potential (Eh) was measured with a platinum-calomel electrode combination. Under the test conditions, Eh values of the medium stayed below −350 mV. Addition of titanium-citrate buffer maintained this value below −550 mV. The pH of the medium generally stayed between 6.7 and 7.0. A drop in pH below 6.7 was taken as failure of acid utilization.

Methods of analysis. Analyses were made to determine gas composition, volatile acids, cellulose breakdown, and sulfate and sulfide content of the fermenter liquids. Gas composition was determined by the method of van Huysesteen (31) by using a Forapak T column (½ inch by 10 feet [ca. 6.35 mm by 304.8 cm]) on a Hewlett-Packard 5710A gas chromatograph equipped with a model 3380A integrator. The column was held at 75°C, and the thermal conductivity detector was held at 150°C. Helium was used as a carrier gas at a flow rate of 40 ml/min. Volatile acids were determined by the method of Ackman (1) by using a Hewlett-Packard 5712A chromatograph equipped with an automatic sampler, a model 3380A integrator, and a Chromosorb 101 column (½ inch by 5.5 feet [ca. 3.18 mm by 167.64 cm]). The column was held at 180°C, and the single flame ionization detector was held at 350°C. Helium was used as a carrier gas at a flow rate of 35 ml/min. Samples were prepared by adding 0.5 ml of sample to 0.5 ml of an internal stanad containing 2,000 mg of isobutyric acid per liter, and the chromatogram was calibrated with a standard containing 2,000 mg each of acetic, propionic, butyric, and isobutyric acids per liter. This equipment was able to detect and quantitate concentrations of 6 to 10 mg/liter and higher of acetic, propionic, and butyric acids at less than a 2% error. Low quantities of propionic or butyric acids, occasionally detected, were equaled 1:1 (on a molar basis) with acetic acid as acetate equivalents for the purpose of tabulation.

The total contents of each vial were centrifuged at 6,000 × g for 20 min. The sediment was used for the determination of cellulose, whereas the supernatant was used for the determination of sulfates and sulfides. The sediment was suspended in 8% formic acid to lyse the cells (32) and then centrifuged. The pellet was dissolved in 72% H₂SO₄, and the cellulose content was determined by anthrone reagent (12). In some cases, these estimations were checked by the dichromate-oxidation procedure (2) used for the determination of chemical oxygen demand. For the measurement of pure cellulose and cellulose in fermenter liquids, the overall accuracy of anthrone reagent was better than 5% and the accuracy of the dichromate-oxidation procedure was better than 2%. For the estimation of sulfates, the supernatant was treated with a uranium acetate solution to remove proteinaceous materials, and sulfates were precipitated as benzinide sulfate, washed free of benzidine, and determined colorimetrically by using sodium β-naphthaquinone-4-sulfonate (11). Sulfide content was estimated by a titrimetric method (2). In view of the absence of the characteristic black iron sulfide precipitate in the sulfur-depleted fermenter liquids and the unavailability of precipitated
sulfides to anaerobes (14, 15), the insoluble sulfide contents were not determined.

**RESULTS**

Removal of supernatant to reduce the content of sulfate and other soluble sulfur-containing compounds present in the inoculum lowered the cellulolytic activity by about 30%, but had little or no effect on methanogenesis occurring from the reduction of CO₂ or from cellulose that was degraded (Table 1). Return of supernatant to the washed cells or prolonged incubation of washed inoculum in sulfate-containing medium restored the cellulolytic activity almost to the original level. Since the removal of soluble sulfates and sulfur-containing compounds is essential in studying the effects of these compounds on cellulose breakdown to CH₄, washed cells having somewhat lower cellulolytic activity were used in this study. Even this washing procedure did not remove all the sulfates and sulfides, since the amount of these compounds in washed inoculum varied between 0.10 and 0.15 mM expressed as sulfur. However, in the presence of sulfate, the washed inoculum regained the lost activity during 3 weeks of incubation (Table 1).

Removal of sulfate, as well as other sulfur-containing compounds from the medium, depressed the cellulose degradation by over 80% and reduced methane formation from cellulose, but did not affect its ability to convert H₂ and CO₂ present in the gas phase to CH₄ (Table 2). This depression continued during incubation time for up to 4 weeks, but the addition of sulfate to the medium restored the ability of this culture to degrade cellulose. In the initial stages of fermentation (1 to 2 weeks of incubation), rapid degradation of cellulose caused the accumulation of volatile acids up to a 10 mM concentration (expressed as acetic acid) in the medium.

Table 1. Effect of washing cells on cellulose degradation and methane formation by mixed culture

<table>
<thead>
<tr>
<th>Culture</th>
<th>Incubation time (week)</th>
<th>Cellulose breakdown (mM)</th>
<th>Volatile acids* (mM)</th>
<th>CH₄ formed from cellulose**</th>
<th>CH₄ formed from headspace gas (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without washing</td>
<td>1</td>
<td>17.2</td>
<td>18</td>
<td>28.0</td>
<td>1.6</td>
</tr>
<tr>
<td>After washing</td>
<td>1</td>
<td>11.9</td>
<td>16</td>
<td>18.8</td>
<td>1.6</td>
</tr>
<tr>
<td>After washing +</td>
<td>1</td>
<td>17.6</td>
<td>18</td>
<td>28.9</td>
<td>1.6</td>
</tr>
<tr>
<td>supernatant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without washing</td>
<td>3</td>
<td>23.7</td>
<td>&lt;1</td>
<td>58.7</td>
<td>2.5</td>
</tr>
<tr>
<td>After washing</td>
<td>3</td>
<td>20.8</td>
<td>1</td>
<td>47.0</td>
<td>2.3</td>
</tr>
</tbody>
</table>

* Inoculum was maintained in a sulfur-free medium for 8 weeks before using. Medium used in these tests contained 1.75 mM sulfate and all the essential minerals and vitamins.

** Expressed as glucose equivalents.

* Residual volatile acids expressed as acetic acid equivalents.

** Corrected for headspace gas.

* Headspace gas, 80% H₂ and 20% CO₂; 110 ml/vial. Calculations based on stoichiometry, 4H₂ + CO₂ → CH₄ + 2H₂O, at 25°C.

After washing the cells, a volume of supernatant equivalent to that removed was added.
Table 2. Effect of added sulfate and length of incubation on cellulose degradation and methane formation by mixed culturea

<table>
<thead>
<tr>
<th>Additions</th>
<th>Incubation time (week)</th>
<th>Cellulose breakdown (mM)</th>
<th>Total CH4 (mM)</th>
<th>CH4 (mol/mol of glucose)</th>
<th>CH4 formed from headspace gas (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)*</td>
<td>1</td>
<td>1.6</td>
<td>19.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sulfate</td>
<td>1</td>
<td>13.7</td>
<td>59.8</td>
<td>18.8</td>
<td>1.4</td>
</tr>
<tr>
<td>None (control)*</td>
<td>2</td>
<td>2.7</td>
<td>31.2</td>
<td>0.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Sulfate</td>
<td>2</td>
<td>18.4</td>
<td>85.7</td>
<td>33.6</td>
<td>1.8</td>
</tr>
<tr>
<td>None (control)*</td>
<td>3</td>
<td>4.1</td>
<td>33.0</td>
<td>3.5</td>
<td>0.9</td>
</tr>
<tr>
<td>Sulfate</td>
<td>3</td>
<td>21.1</td>
<td>107.1</td>
<td>50.9</td>
<td>2.4</td>
</tr>
<tr>
<td>None (control)*</td>
<td>4</td>
<td>5.3</td>
<td>40.1</td>
<td>6.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Sulfate</td>
<td>4</td>
<td>22.2</td>
<td>113.4</td>
<td>53.6</td>
<td>2.4</td>
</tr>
</tbody>
</table>

a Inoculum, washed cells. Where indicated, Na2SO4 was added to increase sulfur content of medium to 1.75 mM.

b Expressed as glucose equivalents.

c Corrected for headspace gas.
d Headspace gas, 80% H2 + 20% CO2; 110 ml/vial. Calculations based on stoichiometry, 4H2 + CO2 → CH4 + 2H2O at 25°C.

e Incubated medium used as control contained 0.1 mM sulfur in the form of sulfate and sulfide, and all essential minerals and vitamins.

Table 3. Effect of sulfate and titanium-citrate buffer on maintaining reducing environment, cellulose degradation, and methane formation by mixed culturea

<table>
<thead>
<tr>
<th>Additions</th>
<th>Eh (mV)</th>
<th>Cellulose breakdown (mM)</th>
<th>Volatile acids (mM)</th>
<th>CH4 formed (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>After incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (control)</td>
<td>-437</td>
<td>-463</td>
<td>1.7</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Sulfate</td>
<td>-456</td>
<td>-450</td>
<td>10.1</td>
<td>12</td>
</tr>
<tr>
<td>Ti-citrate</td>
<td>-550</td>
<td>-550</td>
<td>1.1</td>
<td>28</td>
</tr>
<tr>
<td>Sulfate + Ti-citrate</td>
<td>-545</td>
<td>-545</td>
<td>3.1</td>
<td>31</td>
</tr>
</tbody>
</table>

a Incubation time, 1 week.
b Inoculated medium used as control contained 0.1 mM sulfur in the form of sulfide and sulfate. Na2SO4 added to increase sulfur content of medium to 1.75 mM, where indicated.
c Expressed as glucose equivalents.
d Total acids, expressed as acetic acid equivalents.
e Methane formed from headspace gas is included.

does not appear to occur as a result of a rise in Eh.

Addition of 1.5 to 1.7 mM sulfur in the form of sulfate, sulfite, thiosulfate, sulfide, or amino sulfur as cysteine and methionine was equally effective in restoring the ability of the culture to degrade cellulose to CH4 (Table 4). Sulfate, thiosulfate, and sulfite are known to act as electron sinks in anaerobic metabolism, and compete with methanogens for hydrogen (4). This competition would not occur where sulfide or cysteine and methionine were present. Since the nature of sulfur-containing compounds did not affect cellulose degradation to CH4, sulfur appears to be an essential nutrient for the mixed culture present in sewage sludge.

Presence of sulfate of up to a 0.85 mM concentration appears necessary for anaerobic degradation of cellulose by mixed culture. Concentrations of up to 1.75 mM had little or no inhibitory effect on cellulose degradation to CH4, but higher concentrations adversely affected the ratio of cellulose degraded to methane formed after 3 weeks of incubation (Fig. 1). However, this microbial ecosystem tolerated sulfate concentrations of up to 12 mM with no more than a 50% loss in the efficiency of conversion of cellulose to methane. Since there is a general agreement that sulfate is reduced to sulfide in mixed cultures (27), the inhibition occurring as a result of higher concentrations of sulfate noted after 3 weeks of incubation (Fig. 1), but not after 1 week of incubation (Table 5), may be due to the accumulation of sulfide in the medium. In Lake Mendota sediments, a sulfate concentration of 1.56 mM has been shown to cause inhibition (19) of methanogenesis, whereas a concentration of 10 mM caused complete inhibition (7). In freshwater sediments, 0.5 to 1.0 mM sulfate concentrations caused complete inhibition (34).
Since little or no information is available on the amount of sulfate present in sewage sludge or on the effects of sulfate on cellulose degradation by the microbial ecosystem that exists in sewage sludge, direct comparison cannot be made. However, the results obtained after 3 weeks of incubation (Fig. 1) agree generally with those reported by Cappenberg (7), MacGregor and Kee- ney (19), and Winfrey and Zeikus (34), indicating that at higher concentrations sulfate inhibits methanogenesis.

At higher concentrations, the extent of inhibition of cellulose degradation and methanogenesis depended on the nature of the inorganic sulfur (Table 5). At a 9 mM concentration, the inhibitory effect on cellulose degradation in-
increased in the following order, sulfate < thiosulfate < sulfite < sulfide. At this level, all inorganic sulfur compounds, except sulfate, lowered cellulose degradation, completely inhibited methane formation from acids, and even reduced the amount of CH₄ formed from headspace gases. For the conversion of H₂ and CO₂ present in the headspace to CH₄, sulfate had maximum inhibitory effect, whereas sulfate had the least. These results agree with the findings of Winfrey and Zeikus (34), indicating that sulfate in higher concentrations (10 mM) inhibits methanogenesis, but differ from those obtained with co-cultures (4), indicating that Methanobacterium strain M.o.H. in association with desulfovibrio can tolerate sulfate levels of up to 20 mM. These differences may be due to metabolic dependence and competition that exists in mixed cultures as well as to differences in experimental design.

At 1.75 mM concentrations, H₂S inhibited methanogenesis, reduced cellulose degradation, and caused the accumulation of volatile acids in the medium as compared to similar levels of sulfate (Table 6). These inhibitory effects of H₂S increased at higher concentrations, and the inhibition was almost 100% at 10 mM. At this level, the formation of methane from CO₂ and H₂ provided in the headspace was also totally inhibited.

**DISCUSSION**

The results indicate that the presence of sulfur in a defined medium is essential for cellulose degradation to CH₄ by a mixed culture obtained from sewage sludge and that more than 0.1 mM sulfur is not required for the reduction of CO₂ to CH₄ when H₂ is available. For cellulose degradation to CH₄, sulfur contents of up to 0.8 mM in the form of sulfate, thiosulfate, sulfite, sulfide, or cysteine and methionine were equally effective. In concentrations higher than 1.75 mM, the inhibition of cellulose degradation to CH₄ depended on the type of sulfur-containing compound and increased in the following order, sulfate < thiosulfate < sulfite < sulfide < H₂S. Among these, sulfite and H₂S had the greatest inhibitory effect on methanogenesis. The high tolerance for sulfate by the ecosystem reported here is probably due to the presence of H₂ in the initial stages of fermentation and presence of acetate after the start of cellulose degradation, both of which have been shown to prevent sulfate inhibition of methanogenesis (33). All methanogenic bacteria so far studied utilize sulfide as a sulfur source and some also use cysteine, but none have yet been found to utilize other forms of sulfur. Sulfide is essential as a sulfur source for at least three species (38). In medium containing sulfate as a sulfur source, sulfide formed from the reduction of sulfate (27) appears to serve as a sulfur source for the methanogens in mixed cultures. However, similar studies have not been made on cellulose-degrading anaerobes. The results reported here are important in developing a medium for optimal degradation of cellulose to methane.

The initial low yields of methane from washed cells in medium containing sulfur appears to occur as a result of the removal of stimulatory compounds present in the supernatant (3, 18, 24, 26, 30) rather than because of destruction of methanogens during the removal of supernatant, rise in Eh (19), or toxicity due to the presence of heavy metals in the absence of sulfide (14). This is indicated by an increase in CH₄ yields from cellulose breakdown to about 80% of the theoretical value, after an increased incubation time. The initial low yields of CH₄ do not appear to be due to a lower number of methanogens present in the inoculum or to a longer doubling time. Had this been the case, the utilization of headspace H₂ and CO₂, of which all known methanogens were able to use, would not have been observed during that initial period. Since this methanogen had been isolated as a pure culture, it is suggested that the addition of other methanogens to the inoculum would have increased the yield of CH₄. The results reported here are important in developing a medium for optimal degradation of cellulose to methane.

**Table 6. Effect of dissolved H₂S in medium on cellulose degradation and methane formation by mixed culture**

<table>
<thead>
<tr>
<th>Sulfur source</th>
<th>Sulfur content (mM)</th>
<th>Cellulose breakdown (mM)</th>
<th>Volatile acids (mM)</th>
<th>CH₄ formed from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cellulose</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(mM)</td>
</tr>
<tr>
<td>No addition</td>
<td>0.10</td>
<td>3</td>
<td>3</td>
<td>3''</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>1.75</td>
<td>19</td>
<td>18</td>
<td>24'''</td>
</tr>
<tr>
<td>H₂S</td>
<td>1.75</td>
<td>16</td>
<td>22</td>
<td>18''</td>
</tr>
<tr>
<td>H₃S</td>
<td>5.25</td>
<td>12</td>
<td>20</td>
<td>12''</td>
</tr>
<tr>
<td>H₄S</td>
<td>10.50</td>
<td>&lt;0.1</td>
<td></td>
<td>&lt;1'''</td>
</tr>
</tbody>
</table>

* Incubation time, 2 weeks.
* Expressed as glucose equivalents.
* Total acids expressed as acetic acid equivalents.
* Corrected for headspace gas.
* Headspace gas, 80% H₂ + 20% CO₂, 110 cm³/vial. Calculations based on stoichiometry 4H₂ + CO₂ → CH₄ + 2H₂O, at 25°C.
* †, ‡ Significantly different at P < 0.01.
ogens use to derive energy (35), would have occurred at a much slower rate. In these tests, the formation of CH₄ from headspace gases began within 1 h after inoculation and was completed within 1 day. The increase in the fatty acid content of the medium during week 1 of incubation may indicate a slow initial growth rate of fatty acid-catabolizing organisms. This slow rate of initial growth may be due to the presence of H₂ in the headspace during the initial 16 to 24 h of incubation. Rise in Eh is not a factor, as the Eh of the samples containing low concentrations (0.8 mM) of sulfur salts was below -300 mV, a value at which methanogenesis has been known to proceed effectively (7, 19). Toxicity due to the presence of heavy metals (14) does not appear to affect these tests, as these metals were present in essential minimum quantities and a doubling of these quantities did not affect the inoculum performance. However, the importance of controlling the Eh of the medium in which small amounts of inocula and no reducing atmosphere are used (19, 23) and removing heavy metals as their sulfides from complex substrates such as sewage sludge (14, 15) is well established.

The inhibitory effects of high levels of sulfate, sulfide, and H₂S on methanogenesis by mixed cultures are well known (4, 7, 19, 34). In sensitivity towards high levels of these compounds, the microbial ecosystem that exists in sewage sludge is comparable to the microbial ecosystem from lake sediments (7, 19, 34), as well as to co-cultures containing sulfate-reducing and methanol-producing bacteria (4). In sulfate-rich medium, poor methanogenesis, in spite of excellent degradation of cellulose, appears to occur as a result of stimulation of acetate metabolizing non-methanogens (33) and lack of availability of H₂ (4, 21, 34). The utilization of acetate in the presence of sulfate via the reaction \( \text{SO}_4^{2-} + \text{CH}_3\text{COO}^- = \text{HS}^- + 2\text{HCO}_3^- \) has a \( \Delta G \) of -47.3 kJ, as compared to the conversion \( \text{CH}_3\text{COO}^- + \text{H}_2\text{O} = \text{CH}_4 + \text{CH}_2\text{O}_3^- \) which has a \( \Delta G \) of -31.0 kJ (27). Due to this more favorable change in free energy, the growth of nonmethanogenic organisms with sulfate reduction may be selectively enhanced. The presence of H₂-using, sulfate-reducing bacteria in the mixed cultures could further curtail the availability of H₂ for methanogenesis (4).

**LITERATURE CITED**


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