Influence of Sulfide Compounds on the Metabolism of *Methanobacterium* Strain AZ

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**Abstract.** Various organic sulfides and inorganic sulfide were studied in respect to their effect on growth and methane production of *Methanobacterium* strain AZ. In mineral, sulfide-free medium, cysteine regulated the specific rate of methane production (optimum concentration = $5 \cdot 10^{-4}$ mole/l). A supplement of sulfide ($10^{-4}$ mole/l) caused an additional stimulation. Coenzyme M** or glutathione could be substituted for cysteine when sulfide was present. Growth was stimulated by CoM and glutathione to the same extent as with cysteine in sulfide-containing media. The concentration of sulfide in cysteine-containing media affected the excretion of amino acids.

**Key words:** *Methanobacterium* strain AZ — Growth and methane formation — Role of cystine, sulfide, CoM and glutathione.

Barker (1940) observed that in the absence of H$_2$S, growth of *M. omelianskii* was delayed or absent and that higher concentrations ($\leq 8$ mg/100 ml) inhibited growth completely. Bryant et al. (1971) showed that in cultures of *M. ruminantium* strain PS (a strain from sewage sludge), sulfide acted as a sulfur source rather than as a reducing agent. In addition, they found that either sulfide or cysteine could serve as sulfur source for *Methanobacterium* strain M.O.H. A supply of cysteine to cultures growing on sulfide increased growth remarkably. Using titanium(III) citrate as a redox buffer, Zehnder and Wuhrmann (1977) demonstrated that cysteine is, independently of its potential activities as a reductant, an essential amino acid for growth of strain AZ. However, addition of sulfide caused a supplementary increase of methane production and growth. The optimum concentration found ($10^{-4}$ mole/l) coincides with the sulfide concentration in the rumen (Hungate, 1966).

The present paper was initiated to investigate the role of sulfhydryl compounds in *Methanobacterium* strain AZ. Various effects on growth and methane formation as well as on amino acid excretion were demonstrated.

**MATERIALS AND METHODS**

**Growth Media.** Stock solutions were prepared as described previously (Zehnder and Wuhrmann, 1977): 1. K$_2$HPO$_4$, 27.2 g in 1000 ml H$_2$O. 2. Na$_2$HPO$_4$, 28.4 g in 1000 ml H$_2$O. 3. Mineral solution: NH$_4$Cl, 6 g; NaCl, 6 g; CaCl$_2$ · 2 H$_2$O, 2.2 g; MgCl$_2$ · 6 H$_2$O, 2 g in 1000 ml H$_2$O. 4. NaHCO$_3$, 80 g in 1000 ml H$_2$O. 5. Trace metal solution: H$_3$BO$_3$ saturated solution, 1 ml; FeCl$_2$ · 2 H$_2$O, 2 g; ZnCl$_2$, 0.05 g; MnCl$_2$ · 4 H$_2$O, 0.5 g; CuCl$_2$ · 2 H$_2$O, 0.03 g; (NH$_4$)$_6$Mo$_7$O$_{24}$ · 4 H$_2$O, 0.05 g; AlCl$_3$ · 6 H$_2$O, 2 g; HCl conc. 1 ml in 1000 ml H$_2$O. 6. Vitamin solution: according to Wolin et al. (1963). 7. Titanium(III) solution 6 · $10^{-2}$ molar (Zehnder and Wuhrmann, 1976). 8. Sulfide solution: Na$_2$S · 9 H$_2$O, 24.02 g in 100 ml H$_2$O.

The mineral medium was prepared as follows: 20 ml of solution (1) and 47 ml of solution (2) were made up with distilled water to 900 ml and sterilized at 120°C. One milliliter of trace metal solution (5) and 1 ml of vitamin solution (6) in 50 ml of mineral solution (3) were added aseptically to the autoclaved medium by filtering through a 0.2 μ membrane filter (Sartorius). If not stated otherwise, 0.08 g of cysteine HCl in 50 ml of solution (4) were supplemented in the same way. After gassing the medium with 20% CO$_2$ and 80% H$_2$ to remove all oxygen, 30 ml of titanium(III) solution were added and the sulfide solution (8) added according to experimental requirements.

The complex medium is identical to the mineral medium with the exception that 2.5 g of yeast extract were dissolved in the mixture of solutions (1) and (2) before autoclaving.

**Culture Technique.** The Hungate technique (1950, 1969) modified by Bryant and Robinson (1961) was adapted for the cultivation of 100 ml cultures in 1 l serum bottles with serum caps (diameter 35 mm). The media were prepared and sterilized in these bottles as described above. Temperature was 33°C, pH was set to 7.2 (PO$_4$ buffer solution no. 1 and 2).
Table 1. Methane formation as a function of sulfide concentration. Mineral medium, cysteine concentration: $10^{-3}$ mole/l, gas phase $H_2\cdot CO_2 = 80:20$, biomass 10 mg dry weight in 100 ml of medium, temperature: 33°C, pH 7.2

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Sulfide (mole/l)</th>
<th>Cumulative CH$_4$ formation, μmoles mg dry weight$^{-1}$</th>
<th>Rate of CH$_4$ formation, μmoles g dry weight$^{-1}$ min$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$10^{-3}$</td>
<td>12.05, 21.88, 22.32, 20.09, 13.84</td>
<td>125, 192, 210, 196, 147</td>
</tr>
<tr>
<td>100</td>
<td>$10^{-4}$</td>
<td>24.11, 39.29, 41.07, 34.38, 29.02</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>$10^{-5}$</td>
<td>36.16, 56.25, 61.16, 58.48, 43.30</td>
<td></td>
</tr>
<tr>
<td>380</td>
<td>$10^{-6}$</td>
<td>49.55, 70.09, 79.46, 70.09, 56.25</td>
<td></td>
</tr>
</tbody>
</table>

Bacterial Growth. The optical density was measured in 1 cm cuvettes at 578 nm using an Eppendorf photometer. Dry weights were determined on Sartorius membrane filters no. 11306 with 0.45 μm pore size.

Harvesting of Cells. The cultures were transferred anaerobically into small serum bottles (120 ml) previously flushed with a mixture of 80% $H_2$ and 20% $CO_2$. The cells were centrifuged under slight overpressure for 20 min at 1500 g, and the supernatant blown out by the gas mixture.

Amino Acid Analysis. 5 ml samples were taken from culture vessels with sterile disposable syringes. After acidification to pH 2 with HCl conc., they were centrifuged (10 min, 30000 g). Neutral and acid amino acids in the supernatant were determined by column chromatography on a Biocal Instrument AA analyzer.

Gas Measurements. Methane was detected with a Gow Mac gas chromatograph equipped with a porapak Q (80/100 mesh) column and a molecular sieve (80/100 mesh) column connected to a thermal conductivity detector. Samples were taken with a sterile gas-tight syringe. Absolute gas content was calculated on the basis of manometric pressure measurements.

Coenzyme M. Generously supplied by Prof. R. S. Wolfe. All chemicals were obtained from commercial sources.

RESULTS

Effect of Sulfide on Methane Formation

Addition of various amounts of sulfide to cultures in mineral medium containing cysteine as sole sulfur source caused significant differences in the rate of methanogenesis within about 100 min (Table 1). This is a very fast response when compared with the generation time of approx. 58 h for strain AZ in this medium. The optimum concentration of inorganic sulfide for this stimulation was $10^{-4}$ mole/l which confirms the previous findings of Zehnder and Wuhrmann (1976).

Effect of Cysteine on Methane Formation

Cells from cultures grown in mineral medium were harvested anaerobically, washed twice with oxygen-free phosphate buffer (pH 7.2, 1/75 mole/l) and incubated in mineral medium devoid of sulfide and cysteine. For about two generation times, the growth rate remained unchanged, i.e. corresponded to cultures in cysteine-containing media which indicates the presence of a certain cysteine pool in the organisms. Once this reserve was exhausted, growth stopped. At this stage, the cells were harvested and transferred to another sulfur-free medium. Addition of cysteine to such sulfur-impoverished cultures revealed a clear dependence of the specific methane production on cysteine concentration (optimum at $5 \cdot 10^{-4}$ mole/l, Fig. 1). A supply of sulfide ($10^{-4}$ mole/l) in addition to cysteine increased methane formation significantly. Without cysteine, i.e. in the absence of growth, the remaining methanogenesis (which can be considered as endogenous methane formation) is independent of the presence of sulfide in the medium.

Effect of Methane Formation of Compounds Chemically Related to Cysteine

Five sulfide-containing organic compounds (in parenthesis the final concentrations): homocysteine ($10^{-4}$ mole/l), S-methylcysteine ($10^{-4}$ mole/l), glutathione ($10^{-4}$ mole/l), dimethylsulfide ($10^{-4}$ mole/l) and coenzyme M (HS-CoM, 15 ng per ml medium) were tested for their ability to enhance methane production by sulfur-depleted cells. The effects were compared with cultures in cysteine-containing media ($5 \cdot 10^{-4}$ mole/l) and control cultures without any sulfur addition. In the absence of sulfide, cysteine was the only compound to stimulate methanogenesis. In the presence of sulfide ($10^{-4}$ mole/l), CoM and glutathione accelerated methane formation (Fig. 2) although to a lesser extent than cysteine. The other compounds had no effect. Whether this might have been due to lack of uptake by cells was not examined.