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Different K_s Values for Hydrogen of Methanogenic Bacteria and Sulfate Reducing Bacteria: An Explanation for the Apparent Inhibition of Methanogenesis by Sulfate

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Abstract. *Desulfovibrio vulgaris* (Marburg) and *Methanobrevibacter arboriphilus* (AZ) are anaerobic sewage sludge bacteria which grow on H_2 plus sulfate and H_2 plus CO_2 as sole energy sources, respectively. Their apparent K_S values for H_2 were determined and found to be approximately 1 μM for the sulfate reducing bacterium and 6 μM for the methanogenic bacterium. In mixed cell suspensions of the two bacteria (adjusted to equal V_{max}) the rate of H_2 consumption by *D. vulgaris* was five times that of *M. arboriphilus*, when the hydrogen supply was rate limiting. The apparent inhibition of methanogenesis was of the same order as expected from the different K_S values for H_2. Difference in substrate affinities can thus account for the inhibition of methanogenesis from H_2 and CO_2 in sulfate rich environments, where the H_2 concentration is well below 5 μM.

Key words: *Desulfovibrio vulgaris* – *Methanobrevibacter arboriphilus* – K_S values for H_2 – Methanogenesis – Sulfate reduction – Competition for H_2

Methane formation is generally absent in marine sediments, when the sulfate concentration is high (Jørgensen 1980; Martens and Berner 1974; Oremland and Taylor 1978). Methanogenesis is inhibited by the addition of sulfate to freshwater sediments (Winfrey and Zeikus 1977), and to mixed cultures of sulfate reducing and methanogenic bacteria (Abram and Nedwell 1978a). The apparent inhibition of methane formation by sulfate has been attributed to a variety of factors including hydrogen sulfide inhibition (Cappenberg 1974), kinetic competition for substrates (Abram and Nedwell 1978a, b; Bryant et al. 1977; Mountfort et al. 1980; Winfrey and Zeikus 1977) and thermodynamic considerations (Zehnder 1978).

H_2S at concentrations present in marine sediments was found not to significantly affect methanogenic bacteria (Bryant et al. 1977; Jørgensen 1977; Mountfort and Asher 1979; Scherer and Sahm 1981; Wellinger and Wuhrmann 1977). On theoretical grounds the higher free energy change associated with sulfate reduction to H_2S as compared to CO_2 reduction to CH_4 cannot in principle explain the inhibition (McCarty 1972; Thauer et al. 1977). This leaves competition for substrates as the most likely mechanism.

The competition between sulfate reducers and methanogens in sediments or mixed cultures for H_2 and acetate has been extentively investigated by several workers (Abram and Nedwell 1978a, b; Mountfort et al. 1980; Winfrey and Zeikus 1977). Qualitatively their data indicate that sulfate reducers effectively compete with methanogens for both substrates. A quantitative evaluation of the kinetic parameters involved has not been done, however.

In this communication the K_S values for H_2 of *D. vulgaris* (Marburg) and *M. arboriphilus* (AZ) are reported. Both bacteria were originally isolated from sewage sludge, where they compete for H_2, when sulfate is present.

Material and Methods

The H_2/CO_2 gas mixture (80%/20%), H_2 (99.993%), N_2 (99.996%) and the N_2/CO_2 gas mixture (80%/20%) were from Messer Griesheim (Düsseldorf, FRG). Gas mixtures with H_2 concentrations lower than 80% and a constant CO_2 concentration of 20% were obtained by mixing 80% H_2/20% CO_2 with 80% N_2/20% CO_2. The exact concentration of H_2 in the gas mixtures was determined by gas chromatography as described by Schönheit et al. (1980).

Source of Organisms. The organisms used were *Desulfovibrio vulgaris* strain Marburg (DSM 2119) (Badziong et al. 1978; Brandis and Thauer 1981) and *Methanobrevibacter arboriphilus* strain AZ (DSM 744) (Zehnder and Wuhrmann 1977). Both bacteria were originally isolated from similar habitats, the anaerobic sewage treatment plants in Marburg (*D. vulgaris*) and in Zürich (*M. arboriphilus*). *M. arboriphilus* was obtained from the German Collection of Microorganisms (Göttingen, FRG) (DSM).

Growth of Desulfovibrio vulgaris. The sulfate reducer was grown on H_2 and sulfate as sole energy source in a sulfate limited chemostat with a dilution rate of 0.16 h^{-1}. The chemostat culture condition will be described in details in a later publication. Cells were collected overnight on ice and harvested by centrifugation for 10 min at 7,000 × *g*. The cells were resuspended and washed twice in 45 mM NaH_2PO_4, 5 mM K_2HPO_4 buffer pH 6.9 containing 1 mM $MgCl_2$ and were finally suspended in the same buffer at about 20 mg cells/ml (dry weight) and stored on ice under 80% H_2/20% CO_2 until use.

Growth of Methanobrevibacter arboriphilus. The methanogen was grown on H_2 and CO_2 at 35° C in a 500 ml glass fermenter as described previously (Schönheit et al. 1980). The medium (250 ml per fermenter) contained per liter: 50 mmol KH_2PO_4,

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0.2 mmol $MgCl_2$, 0.5 mmol nitrolotriacetate, 50 µmol $FeCl_2$, 5 µmol $NiCl_2$, 1 µmol $CoCl_2$, 1 µmol Na_2MoO_4, 1 g yeast extract and 20 µmol resazurin. The pH of the medium was adjusted to 7.0 with 8 ml 1 M Na_2CO_3. After the addition of the Na_2CO_3 solution the medium was gassed with 80% H_2/20% CO_2, 0.2% H_2S for 25 min and inoculated. After inoculation (10%, v/v) the culture was continuously stirred with a teflon coated stirring bar at 1,100 rpm and gassed with 80% H_2, 20% CO_2, 0.2% H_2S at a rate of 250 ml/min via a microfilter candle, porosity 3 (Schott, Mainz, FRG). Growth was followed by measuring the absorbance at 578 nm. A $\Delta A_{578} = 1$ corresponded to a cell concentration of 410 mg cells (dry weight)/l.

Cell suspensions of *M. arboriphilus* were prepared by the following procedure: Portions of 50 ml of an exponentially growing culture ($\mu = 0.14\ h^{-1}$) were transferred anaerobically to 120 ml centrifuge tubes and centrifuged at 2,000 × *g* for 5 min at 4° C. The cells were washed with 10 ml 45 mM NaH_2PO_4/5 mM KH_2PO_4 buffer pH 6.9 containing 1 mM $MgCl_2$, 5 mM dithiothreitol (DTE) and 20 µM resazurin, suspended in the same buffer at a concentration of 10 mg bacterial dry weight per ml and stored under 80% H_2/20% CO_2 on ice until use.

Determination of K_S for H_2. The apparent K_S for H_2 was determined from the rate of H_2 consumption by cell suspensions. The H_2 concentration was measured with a hydrogen electrode (Clark type electrode YSI 4004, Yellow Springs Instruments Co., Yellow Springs, Ohio, USA) according to Wang et al. (1971) and Jones and Bishop (1976). The assay was performed at 35° C in a 5 ml glass electrode cuvette filled with 4 ml anaerobic phosphate buffer (45 mM NaH_2PO_4, 5 mM KH_2PO_4 pH 6.9, containing 5 mM DTE plus 5 mM Na_2SO_4 in the case of *D. vulgaris*) which had been previously saturated with 10% H_2/20% CO_2. The reaction was monitored after the addition of the bacteria under continuous stirring.

Determination of H_2S and CH_4 Production Rates. The H_2S production by cell suspensions of *D. vulgaris* was determined at 35° C in 9 ml sealed serum bottles containing 4 ml of 45 mM NaH_2PO_4, 5 mM KH_2PO_4 buffer pH 6.9, 5 mM DTE and 20 µM resazurin. After the addition of the cells and 15 µmol of sulfate (see below) the solution was gassed with H_2/CO_2 (H_2 concentration as indicated, CO_2 concentration = 20%) at a gassing rate of 250 ml/min, under continuous stirring with a magnetic stirring bar. The hydrogen sulfide formed was quantitatively trapped as ZnS in zinc acetate solution in a connected serum vial (Fig. 1) and determined as described by Badziong and Thauer (1978). The CH_4 production by cell suspensions of *M. arboriphilus* was determined in the absence of sulfate under the same conditions as described for H_2S production of *D. vulgaris*. The CH_4 formation rates were determined by measuring both the concentration in a connected serum vial and the flow rate of the outcoming gas (see Fig. 1). Methane was measured by gas chromatography on a Varian Aerograph Model 2700 with flame ionisation detection (Schönheit et al. 1980).

In competition experiments both bacteria were mixed in the same serum bottle (mixed arrangement). As a control the two bacteria were incubated in separate vials, which were connected by tubing such that the gas mixture (H_2/CO_2) first passed through the serum bottle with *D. vulgaris* and then that with *M. arboriphilus* (sequential arrangement).

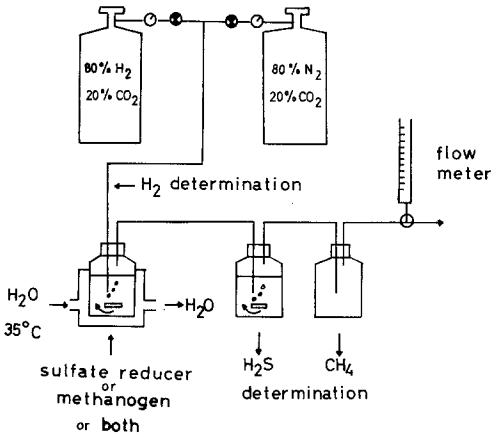

Fig. 1. Scheme of the experimental set-up for determination of H_2S and CH_4 production rates

Table 1. Comparison of the steady state rates of H_2S and CH_4 production by *D. vulgaris* and *M. arboriphilus*

Experiment	Percent H_2 in the incoming gas					
	80%		10%		3%	
	Rate[a]	%	Rate	%	Rate	%
D. vulgaris (alone)	0.65	100	0.50	77	0.23	35
M. arboriphilus (alone)	0.72	100	0.50	75	0.15	20
D. vulgaris plus *M. arboriphilus* (sequential)[b]	0.65	100	0.50	77	0.23	35
	0.67	100	0.54	80	0.18	27
D. vulgaris plus *M. arboriphilus* (mixture)	0.61	100	0.38	63	0.15	25
	0.70	100	0.21	31	0.036	5

[a] Rate is µmol H_2S or $CH_4 \times min^{-1}$. The amount of cells used varied between 0.2 – 0.25 mg dry weight/ml of *M. arboriphilus* and 1 – 1.5 mg dry weight/ml of *D. vulgaris*, depending on the specific activity of the particular cell suspension

[b] The arrangement of the experiments was as described under Materials and Methods

Independent of the arrangement (separate, mixed, or sequential) the two bacteria showed a few minutes lag before constant rates of H_2S and CH_4 production were observed. The data given in Table 1 and Fig. 3 represent the rates after this lag period.

Results

1. Apparent K_S Values for H_2 in Cell Suspensions

The rates of H_2 oxidation by CO_2 and SO_4^{2-} in cell suspensions of *M. arboriphilus* and *D. vulgaris*, respectively, were measured as a function of the H_2 concentration in solution. The concentration of both bacteria in the assay was chosen to give approximately the same rate of H_2 uptake at high H_2 concentration (equal V_{max}). Typical kinetics of H_2 consumption are given in Fig. 2. It can be seen that the

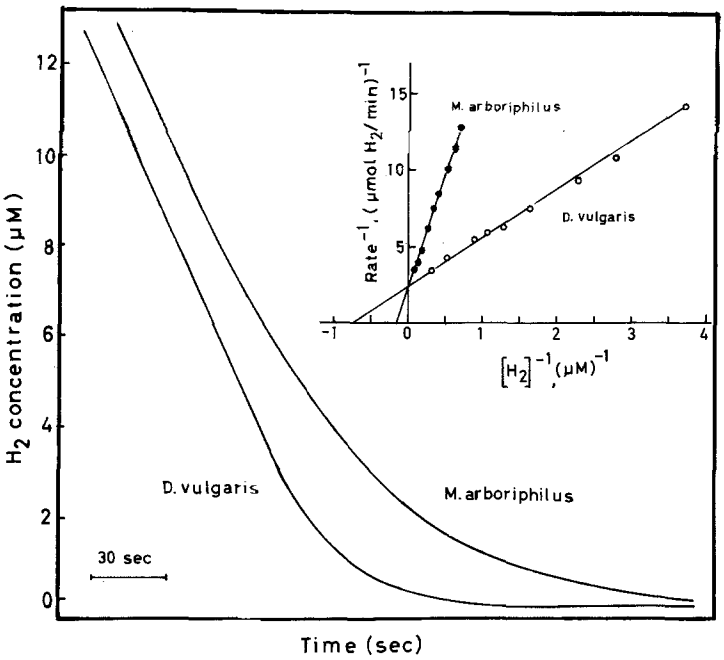

Fig. 2
Hydrogen oxidation by M. *arboriphilus* or *D. vulgaris*. Progress curves for H_2 oxidation, monitored with the hydrogen electrode. The overall response time of the instrument to give full signal was about 5 s (Jones and Bishop 1976). The amount of both bacteria in the reaction vessel were adjusted such as to approach the same V_{max} (2.0 and 0.2 mg dry wt. of *D. vulgaris* and *M. arboriphilus*, respectively). The inset shows a double reciprocal plot of the rate of H_2 oxidation (determined from the slope of the progress curve) versus the H_2 concentration

progress curve for the sulfate reducing bacterium was linear to a lower H_2 concentration than for the methanogen, indicating that the sulfate reducing bacterium had a lower K_S value for H_2. The K_S values were calculated from the double reciprocal plot shown in the inset. The rates at various concentrations of H_2 were determined from the slope (tangent) of the progress curve at the corresponding H_2 concentration. The calculated K_S values were 1.3 μM for *D. vulgaris* and 6.6 μM for *M. arboriphilus*.

2. *Competition for H_2 in Mixed Cell Suspensions*

The reduction of both CO_2 and SO_4^{2-} to CH_4 and H_2S, respectively, consumes 4 H_2; therefore the product formation can be taken as direct measure of H_2 uptake by the individual organisms. The rate of H_2S and CH_4 production in separate cell suspensions of *D. vulgaris* (alone) and of *M. arboriphilus* (alone) was first determined at different H_2 concentrations in the incoming gas (Table 1). The gassing rate was kept constant at 250 ml/min. At a H_2 concentration of 80% the rates of production of H_2S and CH_4 were linear with respect to time and proportional to the cell concentration up to 6 mg dry weight/ml for *D. vulgaris* and 0.2 mg dry weight/ml for *M. arboriphilus* (data not shown). The experimental conditions were chosen such that the supply of H_2 to the cells was not rate limiting at 80% H_2 in the gas. When the partial pressure of H_2 in the incoming gas was lowered to about 3%, the rate of H_2S and of CH_4 production was only 35% and 20%, respectively, of that observed at 80% H_2 (Table 1). The production of H_2S and CH_4 was still linear with respect to time, but the rates were now almost independent of the cell concentration. This indicated that hydrogen transfer into the liquid by diffusion rather than the total supply of hydrogen in the gas became the rate limiting step at low hydrogen partial pressure.

Next the rates of H_2S and CH_4 production in mixed cell suspensions of *D. vulgaris* and of *M. arboriphilus* were measured [Table 1 (mixture)]. The cell concentrations were chosen such as to give equal V_{max}. With 80% H_2 in the incoming gas the same rates were observed in the mixture as when the bacteria were assayed separately and the rate ratio H_2S/CH_4 was about 1. However, when the H_2 supply was made limiting by lowering the partial pressure of H_2 to 3% an apparent inhibition of CH_4 formation was observed. The rate ratio H_2S/CH_4 increased from 1 at 80% H_2 to about 5 at 3% H_2 (Fig. 3). The ratio decreased again to 1, when H_2 was brought back to 80% (not shown) indicating that the observed inhibition was reversible. When the bacteria were incubated separately the rate ratio remained near 1 down to a H_2 concentration of 10% and increased only to about 1.7 at 3% H_2 (Fig. 3).

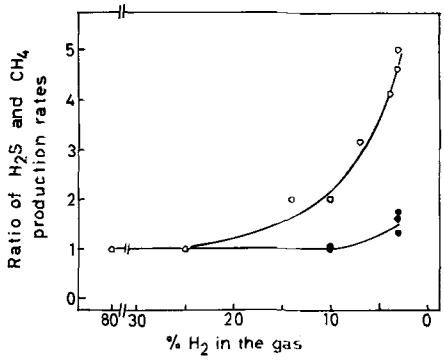

Fig. 3. Competition for H_2 between *M. arboriphilus* and *D. vulgaris* in cell suspensions. The ratio of the rates of H_2S- and CH_4-production is plotted as a function of decreasing H_2 partial pressure in the incoming gas. ●, Separate arrangement; ○, mixed arrangement (see Material and Methods)

The control with the sequential arrangement, where the bacteria were incubated in two separate, but connected vials gave the same rates as when they were gassed separately (Table 1). This showed that the apparent inhibition of methane formation observed in mixed cell suspensions at low H_2 partial pressure is due to competition for dissolved H_2 rather than to inhibition of the methanogen by H_2S produced by the sulfate reducer.

Discussion

An important result of this study is that the apparent K_S value of *D. vulgaris* for H_2 is lower by a factor of 5 than that of *M. arboriphilus*. Attempts have been made earlier to measure the K_S value for H_2 of methanogenic systems. Hungate et al. (1970) estimated the K_S value for rumen fluid and for a pure culture of *Methanobrevibacter ruminantium* (formerly *Methanobacterium ruminantium* M 1), to be in the order of 1 – 10 μM H_2, which is in good agreement with our measurement of about 6 μM for *M. arboriphilus* in cell suspension. Strayer and Tiedje (1978) found the K_S value for H_2 in freshwater lake sediments to be about 15 μM. Schönheit et al. (1980) determined the apparent K_S value for H_2 in the gas phase for a growing culture of *Methanobacterium thermoautotrophicum* (Marburg) to be about 20 % H_2, which does not reflect the actual concentration of dissolved H_2. Under conditions where H_2 is being rapidly utilized by a growing culture, the H_2 in solution cannot be in equilibrium with the gas phase. Comparison of the apparent K_M of the uptake hydrogenase for H_2 in *M. thermoautotrophicum* (20 μM; Fuchs et al. 1979) indicates that the H_2 concentration in the medium can be only one fourth of the equilibrium concentration. From the kinetics of CH_4 production when external H_2 was added to an anaerobic sewage sludge digester, Kaspar and Wuhrmann (1978) determined a K_S value for H_2 of about 10 % in the gas phase (10.5 KPa = 0.105 atm). Here the heterogeneity of the system makes it difficult to assign the K_S for H_2 to a certain bacterial group. However, the K_S value of about 1 μM for *D. vulgaris* reported here is the first time that the K_S value for H_2 has been determined for a sulfate reducing bacterium.

The K_S values for H_2 found in the present work are in good agreement with what might be expected from the H_2 concentration of about 1 μM found in rumen and freshwater sediments (Hungate 1967; Robinson et al. 1981; Strayer and Tiedje 1978) and less than 10 Pa (10^{-4} atm) H_2 partial pressure in sewage sludge digester gas (Kasper and Wuhrmann 1978). In mixed cultures of H_2 producing and H_2 utilizing bacteria taking part in interspecies H_2 transfer the H_2 partial pressure has been found to lie the range of 10^{-4} to 10^{-3} atm (Bryant et al. 1977; McInerney and Bryant 1981).

Veldkamp and Jannasch (1972) have shown that minor differences in K_S values for a substrate are sufficient to allow one organism to outgrow the other in a substrate limited chemostat. The presently available evidence indicates that sulfate reducing bacteria may in general have a lower K_S for H_2 than methanogenic bacteria. By analogy with the work of Veldkamp and Jannasch (1972) the difference in substrate affinity found in the present work would be sufficient to explain the apparently competitive relationship between these bacteria in natural ecosystems.

The reasons why sulfate reducing bacteria should in general have a lower apparent K_S value for H_2 than methanogenic bacteria can only be speculative. One possibility is that the periplasmic location of the hydrogenese of sulfate reducing bacteria (Badziong and Thauer 1980; Bell et al. 1974) would result in a lower *apparent* K_S for hydrogenase. This can be rationalized since the cytoplasmic membrane could represent a barrier and under active H_2 oxidation the $[H_2]$ in the cell must be lower than the $[H_2]$ outside, as was suggested by Kristjansson and Hollocher (1980) in the case of N_2O reductase in denitrifying bacteria. Another possibility is that thermodynamics indirectly put a constraint on the kinetic parameters of the biological reactions involved. The free energy change of sulfate reduction to H_2S with H_2 is 151 kJ/mol sulfate whereas that of CO_2 reduction to CH_4 with H_2 is only 135 kJ/mol CO_2. The Haldane equation (Fersht 1977) predicts that K_S of an enzyme is in part determined by the free energy change associated with the particular reaction.

In any case the experiments reported herein show that CH_4 production and sulfate reduction are not mutually exclusive and in the presence of excess H_2 have no effect on each other. When the H_2 supply becomes rate limiting, however, competition does take place. The methanogenic bacteria are not inhibited *per se* by the activity of the sulfate reducing bacteria but have a lower affinity for the common substrate which results in their suppression.

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