Arch Microbiol (1986) 146:181–185

Archives of
Microbiology

Monensin and gramicidin stimulate CH_4 formation from H_2 and CO_2 in *Methanobacterium thermoautotrophicum* at low external Na^+ concentration

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Abstract. Methane formation from H_2 and CO_2 in methanogenic bacteria is a Na^+-dependent process. In this communication the effects of Na^+ ionophores, of uncouplers, and of Na^+/H^+ antiporter inhibitors on methane formation from H_2 and CO_2 were studied with *Methanobacterium thermoautotrophicum*.

1. Na^+ ionophores (the Na^+/H^+ antiporters monensin and lasalocid and the Na^+ uniporter gramicidin) stimulated methanogenesis at low external Na^+ concentrations when the K^+ concentration was high. The ionophores had no effect at high external Na^+ concentrations and were inhibitory at low external K^+ concentrations.

2. Uncouplers (protonophores and valinomycin plus K^+) inhibited methanogenesis at low external Na^+ concentration at both low and high external K^+ concentrations. Inhibition by uncouplers was relieved by the addition of either Na^+ or Na^+ ionophores.

3. Na^+/H^+ antiporter inhibitors (harmaline, amiloride, and NH_4^+) inhibited methanogenesis at low external Na^+ concentration. Inhibition was relieved by the addition of either Na^+ or of the Na^+ ionophores.

The results are discussed with respect to the role of Na transport across the cytoplasmic membrane in methanogenesis from H_2 and CO_2.

Key words: *Methanobacterium thermoautotrophicum* – Na^+ dependent methanogenesis – Na^+/H^+ antiporter – Monensin – Gramicidin – Uncoupler

Methanogenic bacteria require Na^+ for methane formation from H_2 and CO_2, acetate, and CH_3OH (Perski et al. 1982), but not from HCHO and H_2 plus CH_3OH (Blaut and Gottschalk 1985; Blaut et al. 1985). The function of Na^+ is not yet understood. Recently a Na^+/H^+ antiport activity was demonstrated in *Methanobacterium thermoautotrophicum* and evidence was presented for an involvement of the antiporter in the case of methane formation from H_2 and CO_2. Thus, for example, inhibition of the antiporter by harmaline (a competitive inhibitor with respect to Na^+) resulted in an inhibition of CH_4 formation (Schönheit and Beimborn 1985a). The results indicate that Na^+ transport across the cytoplasmic membrane (via the Na^+/H^+ antiporter) is essential for methanogenesis. It was therefore of interest to investigate whether ionophores, which mediate Na^+ transport, stimulate methanogenesis under conditions where the rate of methane formation is controlled by the extracellular Na^+ concentration. The effects of monensin, lasalocid (artificial Na^+/H^+ antiporters) and of gramicidin (a channel forming Na^+ uniporter) were studied.

Monensin, lasalocid and gramicidin have been reported to inhibit growth and CH_4 formation of most methanogens studied so far, including *M. thermoautotrophicum* (Chen and Wolin 1979; Dellinger and Ferry 1984; Hilpert et al. 1981; Jarrell and Sprott 1983a, b; Jarrell and Hamilton 1985; Perski et al. 1982; Wildenauer et al. 1984). In addition, there are reports on the effects of monensin and gramicidin on the pH gradient, the membrane potential and the intracellular ATP concentration in methanogens (Jarrell and Sprott 1983a, b and 1985; Butsch and Bachofen 1984; for a review see Daniels et al. 1984).

Most of the experiments were conducted at low extracellular K^+ concentration. Since monensin and lasalocid also catalyze K^+/H^+ exchange and gramicidin catalyzes electrogenic movement of K^+ in addition to Na^+ (Pressmann 1976; Bakker 1978) inhibition of methane formation by these compounds could be the result of K^+ depletion.

It is shown in this communication that at high external K^+ concentration the Na^+ ionophores can stimulate rather than inhibit methanogenesis.

Materials and methods

Gases (CH_4, H_2, CO_2, N_2, H_2S) and the gas mixture 80% H_2/20% CO_2 were obtained from Messer Griesheim (Düsseldorf, FRG). Monensin was obtained from Calbiochem (Frankfurt/Main, FRG) and gramicidin D from Serva (Heidelberg, FRG). Valinomycin was from Boehringer (Mannheim, FRG). 3,5-Di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF 6847) was a gift from Prof. Hanstein (Bochum, FRG). 3,5,3′,4′-tetrachlorosalicylanilide (TCS) was a gift from Dr. Booth (Aberdeen, UK). Lasalocid and harmaline (1-methyl-7-methoxy-3,4-dihydro-β-carboline) were obtained from Sigma (Taufkirchen, FRG). Amiloride (3,5-diamine-6-chloropyrazinoyl-guanidine-HCl) was a gift from Dr. Mennerich, MSD Sharp & Dohme GmbH (München, FRG). Ethanolic (100%) solutions of sodium ionophores, uncouplers and Na^+/H^+ antiporter inhibitors were used in the experiments. Corresponding amounts of ethanol were added to the controls. Tetra (U-^{14}C) phenylphosphonium (^{14}C-TPP$^+$) (1160 MBq/mmol), and ^3H_2O (1.85 MBq/ml) were obtained from Amersham Buchler (Braunschweig, FRG), and ATP monitoring reagent from LKB Instruments GmbH

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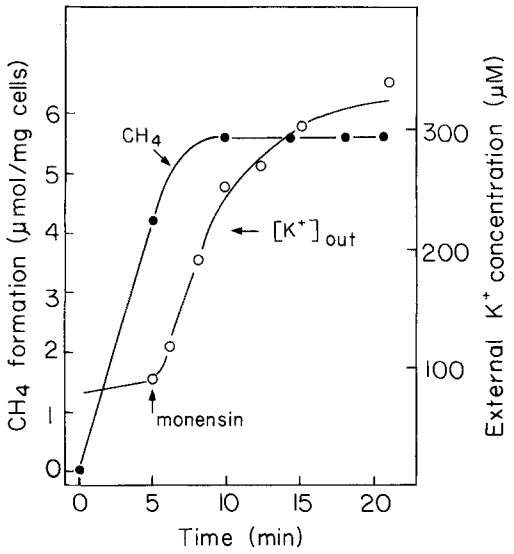

Fig. 1. The effect of monensin on methane formation from H_2 and CO_2 and on K^+ efflux in cell suspensions of *Methanobacterium thermoautotrophicum*. The assay was performed at 65° C in a 150 ml sealed glass flask filled with 5 ml assay mixture containing per ml: 50 µmol Tris. 5 µmol dithioerythritol (DTE). 20 nmol resazurin, 0.4 mg cells (dry weight), 0.15 µmol NaCl and 0.15 µmol KCl. The gas phase was 80% H_2/20% CO_2 at a pressure of 200 kPa (final pH = 7). The reaction was started by the addition of cells. After 5 min 50 nmol monensin per mg cells were added and methane formation and the extracellular K^+ concentration were followed

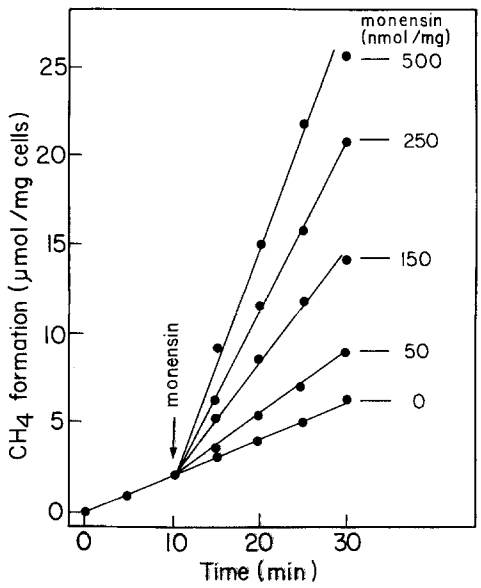

Fig. 2. Stimulation of methane formation from H_2 and CO_2 in cell suspensions of *Methanobacterium thermoautotrophicum* by monensin. The assay conditions were as in Fig. 1. The assay mixture contained per ml: 50 µmol Tris, 5 µmol DTE. 20 nmol resazurin, 0.4 mg cells. 0.15 µmol NaCl and 25 µmol KCl. The reaction was started by the addition of cells and after 10 min 0–500 nmol monensin per mg cells were added

(Gräfelfing, FRG). *Methanobacterium thermoautotrophicum* strain Marburg (DSM 2133) was from the Deutsche Sammlung von Mikroorganismen (Göttingen, FRG).

Preparation of cell suspensions

M. thermoautotrophicum was cultured in a 500 ml glass fermenter containing 250 ml mineral salt medium; H_2 and CO_2 were the sole carbon and energy sources, and H_2S the sulfur source as described by Schönheit et al. (1980). The exponentially growing culture (at a cell concentration of 1 g dry weight/l) was cooled down to 0° C while gassed with the $H_2/CO_2/H_2S$ gas mixture. The cells were then harvested anaerobically, washed and finally suspended in 50 mM Tris [(Tris-hydroxymethyl)amino-methane]/HCO_3^- buffer (pH 7.2) containing 5 mM dithioerythritol (DTE), and 20 µM resazurin. The cell suspension (30–40 mg dry weight/ml) was stored at 0° C under a 80% H_2/20% CO_2 gas phase until use.

Determination of CH_4

CH_4 formation by cell suspensions was determined by gas chromatography as described by Schönheit et al. (1980). Detailed descriptions of the assay conditions and of the assay mixtures are given in the legends to the figures.

Determination of the membrane potential ($\Delta\Psi$)

$\Delta\Psi$ across the cytoplasmic membrane of *M. thermoautotrophicum* was determined by measurement of the transmembrane equilibrium distribution of ^{14}C-TPP^+ according to Rottenberg (1979). The procedure for the determination of the distribution and unspecific binding of TPP^+ to the cells was as described previously (Schönheit et al. 1984; Schönheit and Beimborn 1985b).

Determination of ATP

The intracellular ATP concentration was determined using the luciferin/luciferase system as described by Schönheit and Beimborn (1985b).

Determination of Na^+ and K^+

Na^+ and K^+ were determined by atomic absorption spectroscopy as previously described (Schönheit and Perski 1983).

Results

The effects of sodium ionophores on methanogenesis from H_2 and CO_2 (monensin, gramicidin, lasalocid)

Monensin catalyzes Na^+/H^+ and K^+/H^+ exchange with a selectivity of Na^+:K^+ of 10:1 (Pressmann 1976; Bakker 1978). The addition of this Na^+ ionophore to cell suspensions of *Methanobacterium thermoautotrophicum* in a Tris/HCO_3^- buffer, pH 7, both low in Na^+ (<0.2 mM) and K^+ (<0.2 mM) caused complete inhibition of methane formation from H_2 and CO_2. K^+ efflux from the cells was observed under these conditions (Fig. 1). No change of the external Na^+ concentration could be detected by means of atomic absorption spectroscopy, which does not exclude that a Na^+ efflux has taken place, since the intracellular Na^+ concentration (5–50 mM) is lower than 10% of that of K^+ (700–1000 mM). The inhibition of methane formation by monensin was probably due to K^+ depletion of the cells (or to the coupled H^+ influx), since it could be prevented by increasing the external K^+ concentration from

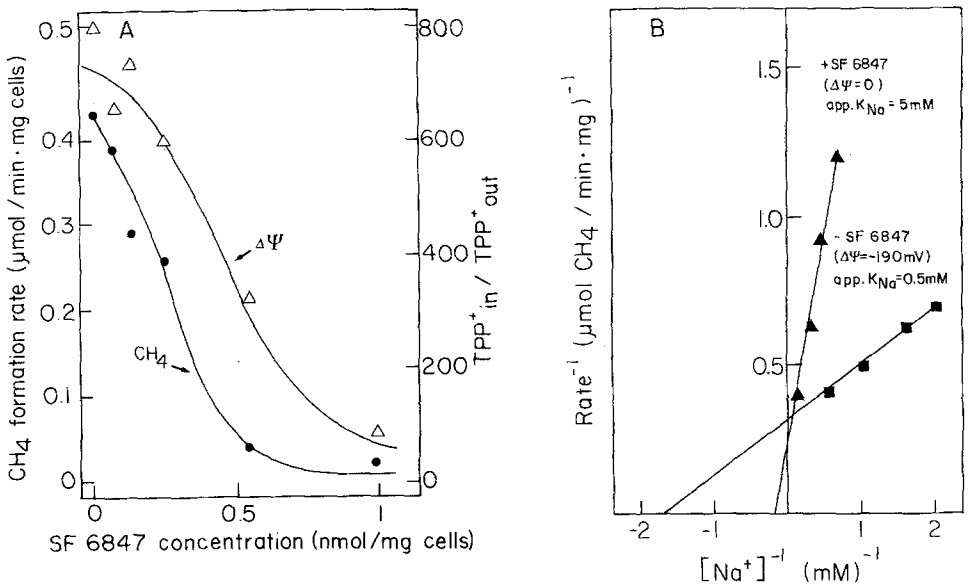

Fig. 3A, B. Inhibition of methane formation from H_2 and CO_2 in cell suspensions of *Methanobacterium thermoautotrophicum* by the protonophore SF 6847. The assay conditions were as in Fig. 1. **A** The assay mixture contained per ml: 50 µmol Tris, 5 µmol DTE, 20 nmol resazurin, 0.4 mg cells, 5 µl ^3H_2O (1.85 MBq/ml), 0.5 nmol ^{14}C-TPP^+ (1160 MBq/mmol), 0.15 µmol KCl, 0.15 µmol NaCl and 0–0.4 nmol SF 6847. The methane formation rates and the TPP^+ accumulation ratios (TPP^+_{in}/TPP^+_{out}) were each determined in separate experiments. A TPP^+_{in}/TPP^+_{out} of 800 is equivalent to a membrane potential of 190 mV (inside negative). **B** The assay mixture contained per ml: 50 µmol Tris, 5 µmol DTE, 20 nmol resazurin, 0.4 mg cells, 25 µmol KCl, 0.1–25 µmol NaCl, and 0 or 0.4 nmol SF 6847. The methane formation rates in the absence or in the presence of SF 6847 were determined at different external Na^+ concentrations. From the double reciprocal plots of the methane formation rate versus the Na^+ concentration the app. K_{Na} values in the absence (app. K_{Na} = 0.5 mM) or in the presence of SF 6847 (app. K_{Na} = 5 mM) and V_{max} values (3 µmol/min × mg) were calculated

<0.2 mM to 25 mM. Below pH 6 monensin inhibited methanogenesis even at high K^+ concentration. Thus in order to study Na^+-specific effects of monensin on methanogenesis, K^+ concentration higher than 25 mM and a pH of 7 were used.

Figure 2 shows that at an external K^+ concentration of 25 mM monensin stimulated rather than inhibited methane formation, when the external Na^+ concentration was <0.2 mM. Maximal stimulation was observed at a monensin concentration of 500 nmol/mg cells (dry weight). At this concentration the rate of methane formation was about 50% of that observed at saturating Na^+ concentrations. The app. K_m for Na^+ was found to be <0.1 mM in the presence of monensin (500 nmol/mg). In the absence of monensin the app. K_{Na} was 0.5 mM. Monensin had no effect on the methane formation rate at high external Na^+ concentration (25 mM).

Gramicidin D, a channel forming ionophore, catalyzes electrogenic movement of Na^+, K^+ [selectivity $Na^+:K^+$ = 1:1.8, value for gramicidin A (Pressmann 1976; Bakker 1978)] and H^+ across the cytoplasmic membrane. The effects of this ionophore on methanogenesis were similar to those obtained with monensin. The compound inhibited CH_4 formation at low external K^+ by catalyzing K^+ efflux from the cells, and stimulated methane formation at high external K^+ (75 mM) when the external Na^+ concentration was low (<0.2 mM) (data not shown).

Lasalocid catalyzes Na^+/H^+ exchange and K^+/H^+ exchange with a selectivity of $Na^+:K^+$ of 1:3 (Pressmann 1976). The compound also catalyzed a K^+ efflux from *M. thermoautotrophicum* cells at low external K^+ concentration and thereby inhibited methane formation. No inhibition was observed at high external K^+ (50 mM). Under these conditions lasalocid stimulated methane formation at low external Na^+ (<0.2 mM) (data not shown).

The effect of uncouplers on methanogenesis from H_2 and CO_2 (SF 6847, TCS, and valinomycin plus K^+)

Protonophores and valinomycin plus K^+ have been shown to dissipate the membrane potential across the cytoplasmic membrane ($\Delta\Psi$) in *M. thermoautotrophicum* without affecting the rate of methane formation when the Na^+ concentration was 25 mM (Schönheit and Beimborn 1985b). We have now studied the effects of uncouplers on methanogenesis at low Na^+ concentration.

The addition of the protonophore SF 6847 to cell suspensions of *M. thermoautotrophicum* in a Tris/HCO_3^- buffer, pH 7, both low in Na^+ (<0.2 mM) and K^+ (<0.2 mM) resulted in an inhibition of methane formation from H_2 and CO_2 and in a decrease of $\Delta\Psi$ (Fig. 3). Inhibition of methane formation by SF 6847 was observed both at low (<0.2 mM) and at high external K^+ (25 mM). The inhibition by the protonophore could, however, be completely relieved by the addition of Na^+. Double reciprocal plots of the methane formation rate versus the Na^+ concentration with and without the protonophore were linear. From the intercepts on the ordinate and on the abscissa it was calculated that the protonophore increased the app. K_m for Na^+ from 0.5 mM to about 5 mM (25 mM KCl), without significantly affecting V_{max} (Fig. 3B). Similar results to those with the protonophore SF 6847 were obtained with the protonophore TCS and with valinomycin plus K^+ (50 mM) (data not shown). Na^+ had no effect on the membrane potential, since in the presence of the uncouplers $\Delta\Psi$

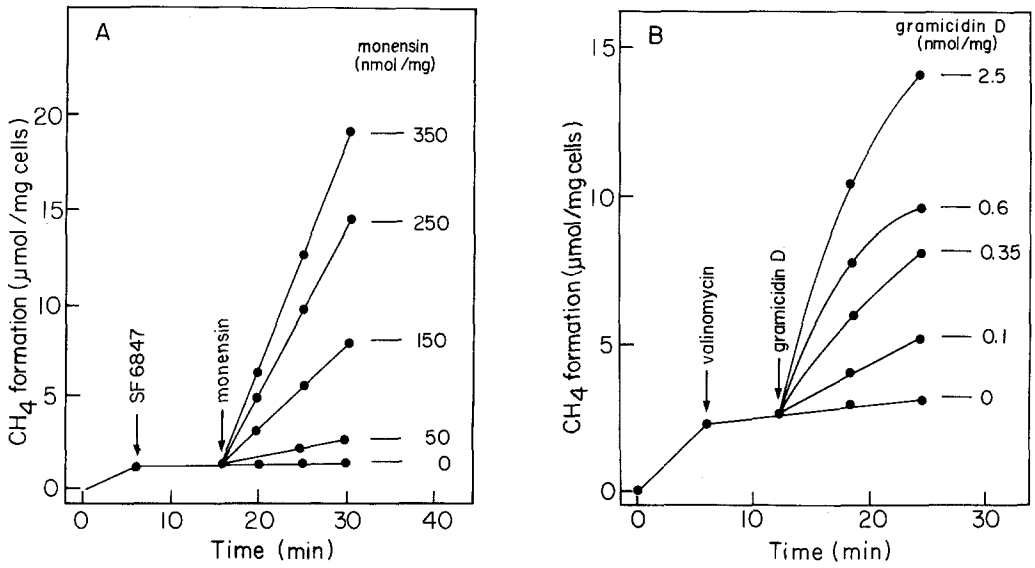

Fig. 4A, B. Stimulation of methane formation from H_2 and CO_2 in cell suspensions of *Methanobacterium thermoautotrophicum* by Na^+ionophores after inhibition by uncouplers. The assay conditions were as in Fig. 1. The assay mixture contained per ml: 50 µmol Tris. 5 µmol DTE, 20 nmol resazurin, 0.4 mg cells, 0.15 µmol NaCl and **A** 50 µmol KCl or **B** 80 µmol KCl. The reaction was started by the addition of cells. After 5 min (A) 5 nmol SF 6847/mg or after 6 min (B) 100 nmol valinomycin/mg, and, after 15 min (A) 0–350 nmol monensin/mg or after 12 min (B) 0–2.5 nmol gramicidin D/mg were added

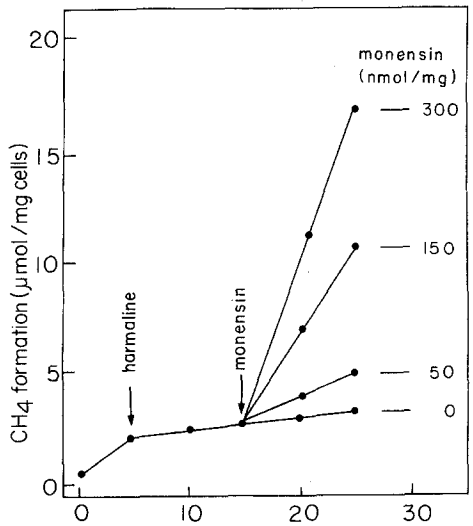

Fig. 5. Stimulation of methane formation from H_2 and CO_2 in cell suspension of *M. thermoautotrophicum* by monensin after inhibition by harmaline. The assay conditions were as in Fig. 1. The assay mixture contained per ml: 50 µmol Tris, 5 µmol DTE, 20 nmol resazurin, 0.4 mg cells, 0.15 µmol NaCl and 50 µmol KCl. The reaction was started by the addition of cells. After 5 min 0.3 mM harmaline and after 15 min 0–350 nmol monensin per mg cells were added

remained essentially zero independent of whether the Na^+ concentration was high or low.

It was found that inhibition of CH_4 formation by protonophores or by valinomycin plus K^+ could be relieved not only by the addition of Na^+ but also by the addition of the Na^+ ionophores monensin, lasalocid and gramicidin, when the external K^+ concentration was high (>25 mM). As shown for monensin (Fig. 4A) and for gramicidin (Fig. 4B) these Na^+ ionophores stimulated methane formation maximally at a concentration of 350–500 nmol/mg cells and 2–3 nmol/mg cells, respectively. The membrane potential remained essentially zero under these conditions.

The effects of the Na^+/H^+ antiporter inhibitors on methanogenesis from H_2 and CO_2 (harmaline, amiloride, NH_4^+)

Harmaline, amiloride and NH_4^+ have been shown to be competitive inhibitors of the Na^+/H^+ antiporter in *M. thermoautotrophicum* (Schönheit and Beimborn 1985a). The addition of 0.3 mM harmaline to cell suspensions of *M. thermoautorophicum* in a Tris/HCO_3^- buffer, pH 7, low in Na^+ (<0.2 mM; 25 mM KCl) caused a 90% inhibition of methane formation from H_2 and CO_2 (Fig. 5); $\Delta\Psi$ decreased from 190 to about 140 mV (inside negative), probably because of the lowered CH_4 formation rate. A similar decrease in $\Delta\Psi$ was observed in the absence of the inhibitor, when the methane formation rate was similarly low as a consequence of limited H_2 supply (gas phase: <10% H_2; see also Butsch and Bachofen 1984).

Inhibition of CH_4 formation by harmaline could be relieved by Na^+ (Schönheit and Beimborn 1985a), and by the addition of the Na^+ ionophores monensin, lasalocid or gramicidin. As shown for monensin (Fig. 5) maximal stimulation of methanogenesis was obtained at 300–500 nmol/mg. Under these conditions the methane formation rate was about 50% of that observed at saturating Na^+ concentrations.

Results similar to those with harmaline were observed with amiloride (0.5 mM) and NH_4^+ (50 mM).

The effects of Na^+ ionophores, uncouplers, and Na^+/H^+ antiporter inhibitors on the intracellular ATP concentration

In most of the experiments on the effects of Na^+ ionophores, uncouplers and Na^+/H^+ antiporter inhibitors, the intracellular ATP concentration was also determined. It was

found that the ATP content was always high (7–9 nmol/mg cells) when the CH_4 formation rate was higher than 0.2–0.4 μmol/min × mg cells, no matter whether the compounds had dissipated the membrane potential or not. The ATP content always decreased below 8 nmol/mg cells, when the rate of methane formation was lower than 0.2 μmol/min × mg cells. A value as low as 1 nmol/mg was observed when the methane formation rate was essentially zero.

Discussion

The sodium ionophores monensin, lasalocid and gramicidin, which catalyze Na^+ transport were found to stimulate rather than inhibit methanogenesis at high K^+ concentration (> 25 mM) when the external Na^+ concentration was low. Under these conditions uncouplers and Na^+/H^+ antiporter inhibitors inhibited methanogenesis from H_2 and CO_2. Inhibition could be relieved by Na^+ or by Na^+ ionophores.

At low external Na^+ concentrations the rate of methane formation is dependent on the Na^+ concentration. The rate dependence followed simple Michaelis-Menten kinetics. The apparent K_m for Na^+ was 0.5 mM (25 mM KCl). With increasing concentrations of the inhibitors, uncouplers and Na^+/H^+ antiporter inhibitors were found to increase the app. K_m for Na^+ from 0.5 mM to approximately 5 mM (shown for protonophores in Fig. 3B). The Na^+ ionophores, in contrast, decreased the app. K_{Na} from 0.5 mM to below 0.1 mM. The effect of the Na^+ ionophores on the K_{Na} was even more pronounced in the presence of uncouplers and of Na^+/H^+ antiporter inhibitors, where the app. K_{Na} decreased from 5 mM to < 0.1 mM (data not shown). The Na^+ ionophores thus exerted their stimulatory effect on methanogenesis at low Na^+ concentration (in the absence and presence of uncouplers or of Na^+/H^+ antiport inhibitors) by lowering the app. K_m for Na^+.

The finding that harmaline (0.3–0.6 mM) increased the app. K_m for Na^+ indicates that Na^+ transport via the Na^+/H^+ antiporter controls the rate of methanogensis from H_2 and CO_2 when the external Na^+ concentration is low.

The stimulatory effects of the Na^+ ionophores and the inhibitory effects of H^+ ionophores on methanogenesis at low external Na^+ cannot be explained at the moment. However, both types of ionophores probably affect, stimulate or inhibit, the transport of Na^+ across the cytoplasmic membrane. At present, studies on Na^+ transport are performed, which have to show, whether this interpretation is correct.

Acknowledgements. The authors thank R. Thauer for helpful discussions. This work was supported by grants from the Deutsche Forschungsgemeinschaft.

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Received May 26, 1986/Accepted July 8, 1986