Methanogenesis and the K⁺ Transport System Are Activated by Divalent Cations in Ammonia-treated Cells of *Methanospirillum hungatei* 

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We describe a K⁺ transport system in *Methanospirillum hungatei* cells depleted of cytoplasmic K⁺ via an ammonia/K⁺ exchange reaction (Sprott, G. D., Shaw, K. M., and Jarrell, K. F. (1984) J. Biol. Chem. 259, 12602–12608). Ammonia-treated cells contained low concentrations of ATP and were unable to make CH₄ or to transport ⁸⁶Rb⁺. All of these properties were restored by CaCl₂, MgCl₂, or MnCl₂, and not by CoCl₂ or NiCl₂. The Rb⁺ transport system had a pH optimum for transport in the range 6.0–7.3 where the transmembrane pH gradient would contribute minimally to the proton motive force. Protonophores at pH 6.3 caused a partial decline in CH₄ synthesis and the ATP content and dramatically collapsed Rb⁺ transport. These and other inhibitor experiments, coupled with the fact that the Rb⁺ gradient was too large to be in equilibrium with the proton motive force alone, suggest a role for both ATP and the proton motive force in Rb⁺ transport. Also, a role for K⁺ in osmoregulation is indicated.

During growth via H₂-dependent CO₂ reduction, methanogenic bacteria exhibit a wide species variation in their cytoplasmic K⁺ contents, but all accumulate K⁺ to concentrations above those in the medium (1–3). This ability to accumulate K⁺ is typical of eubacteria and *Halobacterium* species (see Ref. 4), being linked to functions of enzyme activation, osmoregulation (5), and pH homeostasis (6–13). In the case of the methanogenic grouping of archaeabacteria, little is known about either the function or transport of this important cation (4). It may be relevant that *Methanobacterium thermoautotrophicum* maintains a cytoplasmic K⁺ concentration (3) which corresponds to the 1 M KCl required for maximal activation of the partially purified hydrogenase (14).

During the reduction of CO₂ to CH₄, methanogenic bacteria appear to maintain their cytoplasmic pH slightly acidic (pH 6.6–6.8) and to generate an electrical potential (inside negative) of −120 to −200 mV (15–20). Growth is usually conducted in media buffered near pH 6.8 where the membrane potential is the predominant, or sole, component of the pmf. An ATP pool is maintained in the range of 1–23 nmol/mg protein (4, 21), being especially large in *Msp. hungatei* (21). These factors may be relevant to the bioenergetics of K⁺ movement, since activity of the low affinity, constitutive systems of *Escherichia coli* (22) and *Streptococcus faecalis* (9) requires both ATP and the pmf.

A dramatic ammonia/K⁺ exchange reaction occurs in *Msp. hungatei*, *M.arkeri*, *E. coli*, and *Bacillus polymyxa* (20). We postulated that following diffusion inward of the NH₃ species, a K⁺/H⁺ antiport activity is triggered in a futile attempt to prevent large increases in the cytoplasmic pH (20). A K⁺/H⁺ antiporter model has been used to explain the loss of cytoplasmic K⁺ when weak alkylamines are added to *Vibrio alginolyticus* or *E. coli* (23). Because the K⁺/H⁺ antiporter activity may react differently, or be absent, in different bacteria, this model seems preferred to a simple leak mechanism in explaining the ineffectiveness of ammonia to cause K⁺ efflux from several other methanogens (20). The exchange reaction is likely to affect growth, especially in media of alkaline pH, although it remains unclear whether organisms exhibiting the exchange would be more, or less, sensitive to ammonia toxicity.

The purpose of the present study was to investigate K⁺ transport in *Msp. hungatei* pretreated with ammonia to deplete the cytoplasmic K⁺. During this approach, we found that methanogenic activity was lost, but could be recovered by the addition of Ca⁺⁺ or Mg⁺⁺. Cells so activated were then able to make ATP and to transport K⁺. Methanogenic activity did not result in net H⁺ efflux unless K⁺ was being transported.

**Experimental Procedures**

**Materials**—⁸⁶RbCl, [¹⁴C]butyric acid (13.4 mCi/mmol), [¹⁴C]urea (57.0 mCi/mmol), and [³H]TPMP bromide (56.0 Ci/mmol) were purchased from New England Nuclear of Canada, Lachine, Quebec, J. T. Baker Chemical Co. supplied Dowex 50W-X8 resin. Other reagents were purchased from the sources described before (16).

**Organism and K⁺ Depletion**—*Msp. hungatei* strain GPI was obtained from Patel et al. (24). The conditions for the growth of this organism in a prereduced, defined salts medium were described previously (1). The abbreviations used are: pmf, proton motive force; *Msp.*, *Methanospirillum*; *Ms.*, *Methanosarcina*; *Mb.*, *Methanobacterium*; TPMP⁺, triphenylmethylyphosphonium ion; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; TCS, 3,3',4',5-tetrachlorosalicylanilide; CCCP, carbonyl cyanide m-chlorophenylhydrazone; pHₐ, pH of the medium.

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cells in the late logarithmic to early stationary growth phase were harvested anaerobically (1,4) in sealed centrifuge tubes, containing CO\textsubscript{2}/H\textsubscript{2} (1:4, v/v). Each pellet (3-4 mg dry weight/10 ml of growth medium) was washed once in 0.1 M Hepes buffer adjusted to pH 6.5 with NaOH (30 mM). Anaerobiosis was maintained throughout by using buffer solutions prerduced with a stream of H\textsubscript{2}S (cysteine and CO\textsubscript{2}/H\textsubscript{2}, 1:4, v/v). Each pellet was resuspended in 5 ml of the wash buffer, buffered to pH 6.5 with NaOH, and filtered, using 0.45-μm cellulose acetate filters. Each filter with adhering cells was washed with 0.1 M LiCl, prepared aerobically. No difference in label retention was noted in cases where the prerewed buffer was compared to aerobic LiCl as wash fluid. Filters were counted in water by liquid scintillation spectrometry. Following each analysis, 0.1 ml aliquots of each reaction mixture were counted for calculation of specific activity.

Proton Motive Force—Measurements of the chemical potential using butyric acid and the membrane potential with TPMP\textsuperscript{+} in the presence of tetraphenylboron have been described (20). The internal space measured before the ammonia treatment was used in the case of the chemical potential where a centrifugation method was used, the total (extra- plus intracellular) H\textsubscript{2}O space was determined with urea (20).

Ammonia Extraction—Cell samples of 3-4 mg (dry weight) were filtered as before (20) and washed with 10 ml of 0.1 M LiCl solution. The filters were placed in small glass Petri dishes, cations were extracted with 0.2 ml of hot 1-butanol (1). The cations K\textsuperscript{+}, Ca\textsuperscript{2+}, and Mg\textsuperscript{2+} in 2.5 ml of H\textsubscript{2}O were quantitated by atomic absorption spectrometry.

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ATP Pools—ATP was extracted from Msp. hungatei with cold HClO\textsubscript{4} and analyzed as described before (21).

Methane Synthesis—CH\textsubscript{4} was quantitated in 0.2-ml aliquots of head space gas by gas-liquid chromatography (20).

RESULTS

Cation Activation of \textsuperscript{86}Rb\textsuperscript{+} Transport—Msp. hungatei depleted of K\textsuperscript{+} by ammonia treatment (20) lost the ability to reaccumulate the ion. However, transport activity was restored with sigmoidal transport kinetics if Mg\textsuperscript{2+} was included in the transport buffer, indicating that the divalent ion was an activator. K\textsuperscript{-} depleted cells exposed to Mg\textsuperscript{2+} for 1 h prior to initiating transport displayed typical Michaelis-Menten kinetics, achieving steady state within 30-60 min (Fig. 1). The concentration gradient was 300-fold, and the cytoplasmic concentration of Rb\textsuperscript{+} was very similar to the original K\textsuperscript{+} concentration prior to depletion. Air, which is a potent inhibitor of methanogenesis (4), prevented transport. In K\textsuperscript{-} loaded cells, the uptake of \textsuperscript{86}Rb\textsuperscript{+} was much diminished. It is noteworthy that K\textsuperscript{+} transport in S. faecalis is subject to feedback regulation (9), a property which seems typical of bacteria.

The activation of Rb\textsuperscript{+} transport by Mg\textsuperscript{2+} can be explained through an activation of methanogenesis. Ammonia-treated cells produced little CH\textsubscript{4}, had lost most of their ATP pool, and retained a K\textsuperscript{+} content of only 0.5 mM (Fig. 2). Upon incubation with low concentrations of Mg\textsuperscript{2+}, methanogenesis was restored even though the Mg\textsuperscript{2+} (and Ca\textsuperscript{2+}) content of the cells had been little influenced by the ammonia treatment. We stress that K\textsuperscript{+} and Rb\textsuperscript{+} were absent for those curves showing recovery of CH\textsubscript{4} and ATP synthesis. Also, 16 mM MgCl\textsubscript{2} had only small stimulatory effects on CH\textsubscript{4} synthesis in K\textsuperscript{-} loaded, untreated cells (Table I).

Various divalent ions were tested for their ability to restore CH\textsubscript{4} synthesis and Rb\textsuperscript{+} transport in K\textsuperscript{-} depleted cells. Methanogenesis and Rb\textsuperscript{+} transport in the absence of divalent cations were 6.5 and 2.5 nmol/min/mg, respectively. Exposure of the K\textsuperscript{-} loaded cells to 10 mM of Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, or Mn\textsuperscript{2+} resulted in activities of 217, 185, and 15 nmol Rb\textsuperscript{+}/min/mg. Fe\textsuperscript{2+}, Co\textsuperscript{2+}, and Ni\textsuperscript{2+} chlorides were relatively ineffective.

Sodium can stimulate methanogenesis in several methanogens, including Msp. hungatei (26). However, we show here that CH\textsubscript{4} synthesis is recovered to rates similar to the non-depleted control cells in the absence of added Na\textsuperscript{+} (Figs. 2 and 3). In these cases, contaminating cations in the buffer had been removed with an exchange resin and glassware acid cleaned. We conclude that exogenous Na\textsuperscript{+} need not be provided for CH\textsubscript{4} synthesis to occur in ammonia-treated cells of Msp. hungatei when incubated with 10 mM MgCl\textsubscript{2}. The Km for Na\textsuperscript{+} in methanogenesis decreases if the medium contains low amounts of K\textsuperscript{+} (27). Thus, this apparent discrepancy with claims that Na\textsuperscript{+} is required for CH\textsubscript{4} synthesis in Msp. hungatei (26) may be explained, at least in part, by the absence of environmental K\textsuperscript{+} in our studies. In the presence of MgCl\textsubscript{2}, 25 mM NaCl had no influence on methanogenesis becoming inhibitory at 50 mM. Sodium seemed to have some ability to activate CH\textsubscript{4} synthesis in the absence of Mg\textsuperscript{2+}, but this may be attributed to the contamination by Mg\textsuperscript{2+} of the NaCl solution (15 mM Mg\textsuperscript{2+} present in 50 mM NaCl solutions).
Fig. 2. Influence of Mg²⁺ concentration on recovery of methanogenesis, ATP synthesis, and ³²Rb⁺ transport in K⁺-depleted M. hungatei. For K⁺ depletion, 117 mM ammonia was added to cell suspensions in a 0.1 M Hepes buffer under a gas phase of CO₂/H₂ (1:4, v/v). After incubation for 10 min, the cells were centrifuged and washed three times in Dowex-treated Tris-Cl buffer (50 mM, pH 6.7). The cells were resuspended in the same buffer solution containing the MgCl₂ concentrations shown in the figure and transferred to acid-cleaned serum bottles filled with CO₂/H₂ (1:4, v/v). Cell preparations were incubated at 35 °C, 100 rpm. CH₄ was analyzed by withdrawing 0.2 ml of head space gas after 30 and 60 min. Following the second CH₄ analysis, a 1.0 ml aliquot was used for ATP extraction, a 2.5 ml aliquot used to measure the amounts of Mg²⁺ remaining, and the remaining cells used for ³²Rb⁺ transport, assayed 4 min following the addition of the label.

Fig. 3. Influence of Na⁺ on methanogenesis and on its recovery in ammonia-treated cells. M. hungatei was K⁺-depleted using ammonia. Cell pellets (0.5 g, dry weight) were washed free of ammonia and resuspended into 5.5 ml of Dowex-treated Tris-Cl buffer, as described in the legend to Fig. 2. Each suspension was divided between two serum bottles containing CO₂/H₂ (1:4, v/v), one of which received 10 mM MgCl₂ injected from a 2.5 mM solution stored under CO₂/H₂. To each set of bottles, NaCl was added in a similar fashion, to the concentrations shown in the figure. Incubation was at 35 °C (100 rpm). Rates of CH₄ synthesis were calculated for the period between 30 and 60 min.

Table I

| Cell treatment | NH₄OH (mM) | MgCl₂ (mM) | CH₄ synthesis (nmol/min·mg) | Cytoplasmic K⁺ (mM) |
|----------------|------------|------------|-----------------------------|--------------------|
|                | 0          | 0          | 141                         | 193                |
|                | 35         | 0          | 3.5                         | 10.5               |
|                | 0          | 50         | 174                         | 200                |
|                | 35         | 50         | 173                         | 18.5               |

As expected for a role of methanogenesis as the primary energy yielding reaction in the cell, gas atmospheres other than CO₂/H₂ greatly diminished Rb⁺ transport. The methanogenic rate of K⁺-depleted cells declined by 97% following the replacement of CO₂/H₂ (1:4, v/v) with CO₂/N₂ (1:4, v/v) or N₂ (100%) for 1 h. Rb⁺ uptake in these latter gas phases was decreased 85-90% in either 4- or 30-min assays (not shown).

Ammonia Causes the Cessation of CH₄ Synthesis and K⁺ Efflux by Different Mechanisms—The effects of ammonia on CH₄ synthesis and K⁺ efflux can be distinguished as separate events. Inclusion of 50 mM MgCl₂ during exposure of the cells to ammonia prevented the loss of methanogenic activity, even though the degree of K⁺ efflux was hardly affected (Table I).

Transport Kinetics—In K⁺-depleted cells, Rb⁺ was transported with a Kᵣ of 0.42 mM and Vₘₐₓ of 29 nmol/min·mg dry weight (Fig. 4). K⁺ inhibited Rb⁺ transport competitively; with 1.0 mM KCl included in the transport assay, the Kᵣ increased to 2.0 mM and Vₘₐₓ was 31 nmol/min·mg. No preference for K⁺ over Rb⁺ was seen, since a 45% inhibition in the initial rate of Rb⁺ transport resulted when both ions were 1.0 mM.

K⁺-associated Ion Movements—The ammonia/K⁺ exchange reaction (20) was excluded as a mechanism for Rb⁺ uptake by the necessity for CH₄ synthesis to drive uptake (previous section) and by quantitative ammonia measurements. Following K⁺ depletion, and washing to remove the cytoplasmic ammonia, the cell-associated ammonia concentration was low and remained quite constant during Rb⁺ uptake (Fig. 5).

The K⁺ transport system is electrogenic, resulting in a large decrease in the membrane potential during Rb⁺ uptake (Fig. 6). Since there is nonspecific binding of [³²H]TPMP⁺ by the filter and cells, it was necessary to determine that the TPMP⁺ released during Rb⁺ transport indicated a decline in the membrane potential and not release of the nonspecific component. This was done by comparing the time-dependent efflux of [³²H]TPMP⁺ from cells transporting Rb⁺ to K⁺-loaded cells where Rb⁺ uptake is much less (Fig. 1). It is evident that RbCl when added to K⁺-loaded cells caused relatively small effects (Fig. 6). Although uptake is clearly electrogenic, the apparent complete quenching of the membrane potential in cells actively transporting Rb⁺ is not certain because of the inaccuracy in measuring small membrane potentials.

An electrogenic uptake of K⁺ (or Rb⁺) is associated with the formation of a chemical potential (Table II). Most striking was the inability of K⁺-depleted cells to regulate their cyto-
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**Fig. 4.** Woolf plot of ⁸⁶Rb⁺ transport showing competitive inhibition by K⁺. Methanogenesis in ammonia-treated *Msp. hungatei* was activated by incubation for 1 h in 0.1 M Hepes buffer (pH 6.5) containing 10 mM MgCl₂. Cell suspensions (2.5 ml) contained in 60-ml serum bottles filled with CO₂/H₂ (1:4, v/v), received ³²P-Rb⁺ (1.09 × 10⁶ cpm/pmol), and samples of 0.36 mg, dry weight/0.5 ml were filtered after 20 s, 2.4 and 6 min. When 1.0 mM KC₁ was added as a competing ion, ³²P-Rb⁺ was added immediately thereafter. Transport rates were calculated from the initial slope (usually linear for at least 4 min). Lines of best fit were determined by linear regression analysis. The correlation coefficients (r) are shown in the figure.

**Fig. 5.** The K⁺ transport system is not coupled to ammonia efflux. A series of cell pellets (3.86 mg, dry weight) were K⁺-depleted with ammonia and washed twice in 0.1 M Hepes (pH 6.5) containing 10 mM MgCl₂. Each pellet was resuspended in 5 ml of Mg⁺²-containing buffer and transferred from the centrifuge tube to a 60-ml serum bottle. A gas phase of CO₂/H₂ (1:4, v/v) was used throughout. Cell suspensions were incubated for 1 h, after which [³H]TPMP⁺ was injected through the serum stoppers. Samples of 1.0 ml (0.82 mg, dry weight) were filtered to determine nonspecific binding, and tetraphenylboron ion added. A steady state distribution of [³H]TPMP⁺ was allowed to occur by incubation for 15 min. Samples were again filtered (zero time in the figure), and followed by injections of 1.0 mM RbCl, as indicated. The data as reported were corrected for binding of [³H]TPMP⁺ to the filters and cells, as described above.

**Fig. 6.** Rb⁺ uptake is associated with a decline in the membrane potential. Nondepleted and ammonia-treated cells were washed twice and resuspended in 0.1 M Hepes (pH 6.5) containing 10 mM MgCl₂. Incubation proceeded for 1 h, at which [³H]TPMP⁺ was injected through the serum stoppers. Samples of 1.0 ml (0.82 mg, dry weight) were filtered to determine nonspecific binding, and tetraphenylboron ion added. A steady state distribution of [³H]TPMP⁺ was allowed to occur by incubation for 15 min. Samples were again filtered (zero time in the figure), and followed by injections of 1.0 mM RbCl, as indicated. The data as reported were corrected for binding of [³H]TPMP⁺ to the filters and cells, as described above.

**Table II**

| Added KCl | pH₀ | pH₁ | ΔpH | Chemical potential (mV) |
|---------|-----|-----|-----|------------------------|
| 0       | 5.64 | 5.72 | 0.08 | 4.9                    |
| 2       | 5.64 | 5.56 | 0.08 | 56                     |

The K⁺-depleted cells were washed and activated for 1 h, as described in the legend to Fig. 6. [¹⁴C]Butyric acid was injected into a series of cell suspensions (2.81 mg, dry weight, in 10 ml under CO₂/H₂ (1:4, v/v)) in 0.02 M Hepes (pH 5.6). Samples were incubated for 10 min, then KCl (2 mM final concentration) was injected into half of the samples. Following incubation for 45 min at 35 °C (100 rpm, tubes horizontal), the cells were centrifuged and label distributions determined (see “Experimental Procedures”).

The K⁺-depleted cells transported Rb⁺ best when the pH of the medium was 6.0–7.2 (Fig. 7). A precipitous decline in transport at alkaline pH values closely followed the methanogenic rate reported before (20), although Rb⁺ uptake was less sensitive to acidic pH.

**Inhibitor Effects**—The most potent inhibitors of Rb⁺ uptake were valinomycin, nigericin, and protonophores. Nigericin (20 μM) inhibited initial transport rates by 83% and valinomycin (20 μM) inhibited by 73% (data not shown). Inhibition of methanogenesis by gramicidin is prevented by the outer wall layers (28), explaining the inability of gramicidin (20 μg/ml) to inhibit Rb⁺ transport in this study. Monensin (20 μM) inhibited neither methanogenesis at pH 6.9 (28) nor Rb⁺ transport at pH 6.5 (29).

In separate experiments, we found the methanogenic rate of K⁺-depleted cells to remain constant and unaltered by the injection of KCl (2 mM) to initiate K⁺ transport.

**pH Response**—K⁺-depleted cells transported Rb⁺ best when plasmic pH, even though CH₄ synthesis was fully activated by Mg⁺².

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transport by direct interaction with the carrier (31). To test the null hypothesis that the lack of inhibition by monensin in Methanococcus voltae monensin inhibits CH₄ synthesis, abolishes the pH gradient (inside alkaline), and lowers dramatically the ATP pool. Rather, the lack of inhibition by monensin in Msp. hungatei supports our conclusion that Rb⁺ uptake and methanogenesis (Figs. 2 and 3) do not require added Na⁺. These results tend to exclude any obligatory coupling between Rb⁺ and Na⁺ movements.

Inhibition of Rb⁺ uptake by valinomycin leads to the conclusion that Rb⁺ (K⁺) is not in equilibrium with the membrane potential, as discussed for S. faecalis (9). Measurements of Rb⁺ uptake following 90-min exposures to the label indicated a concentration gradient of about 2300-fold, in the range of 2–10 μM substrate. Distributions of TPMP⁺ were much lower, in the 120- to 180-fold concentration range. The inhibition of CH₄ synthesis by protonophores was less acute than the inhibition to Rb⁺ transport (Fig. 8). Since current evidence favors that both CH₄ synthesis (4, 29, 30) and Rb⁺ transport depend on the pmf, Rb⁺ transport may be gated, as suggested for Alteromonas haloplanktis (7). In this case, the magnitude of the pmf would be sufficient to maintain a decreased rate of CH₄ synthesis, but insufficient for Rb⁺ transport.

In Saccharomyces fragilis, CCCP is thought to inhibit sugar transport by direct interaction with the carrier (31). To test this possibility, Rb⁺ (50 μM) was allowed to accumulate to a steady state value of 48 nmol/mg. Inhibitors were then added and efflux measured after 15 min (not shown). In the case of CCCP (40 μM), only 4 nmol of ⁸⁶Rb⁺/mg was found to efflux, while TCS (20 μM) caused the efflux of 20 nmol of ⁸⁶Rb⁺/mg. Since efflux is likely to occur through the carrier, it seems that TCS does not interfere with carrier activity, while for CCCP it is less clear. Attempts to measure the effects of protonophores on ⁸⁶Rb⁺/K⁺ exchange were not successful, because the exchange was slow and incomplete (Fig. 9).

Acetylene inhibits CH₄ synthesis in Msp. hungatei with the result that ATP pools decline (21). Incubation of K⁺-depleted cells with 116 μM dissolved acetylene for 1 h resulted in a decline in the CH₄ synthesis rate by 4%, a loss of 50% of the ATP pool, but retention of the membrane potential (146 mV). These cells transported Rb⁺ at 35% of the rate found for uninhibited cells, providing correlative evidence for a role for ATP (or a product of ATP) in transport.

Osmoregulation—Glucose does not penetrate the cytoplasm of a number of methanogens (1, 3) including Msp. hungatei (1). Changes in the osmotic strength of the medium through the inclusion of glucose (up to 100 mM) resulted in an increase in the steady state Rb⁺ accumulation, but little change in either initial transport rates or CH₄ synthesis. Further increases in glucose concentration fully inhibited CH₄ synthesis and caused severe inhibitions in Rb⁺ transport (Fig. 10). Previously, we noted that large increases in the osmotic strength of the medium cause K⁺ to efflux from Msp. hungatei (20).

**DISCUSSION**

Exposure of Msp. hungatei to ammonia at alkaline pH results in an ammonia/K⁺ exchange (20) and in the loss of methanogenic activity. Cells washed free of ammonia recover their ability to make CH₄ when exposed to Ca²⁺, Mg²⁺, or

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Fig. 9. Rates of Rb\textsuperscript+ efflux during K\textsuperscript+ transport into cells containing a Rb\textsuperscript+ pool. Cells were depleted of K\textsuperscript+ and methanogenesis activated by incubating 10-min aliquots of cells (4.66 mg, dry weight) in a buffer containing MgCl\textsubscript{2} (see "Experimental Procedures"). RbCl was injected to give either 50 \mu M (panel A) or 25 \mu M (panel B). Samples of 0.5 ml were filtered at the times indicated. After 30 min, parallel reaction mixtures received either 10 mM KCl (final concentration) or served as an untreated control.

Mn\textsuperscript{2+}. This dramatic enhancement of CH\textsubscript{4} synthesis upon exposure to divalent ions does not occur in K\textsuperscript+-loaded cells, showing that ammonia was responsible for the activity loss. To explain these effects, two alternative models should be considered. The loss of methanogenic activity and K\textsuperscript+ efflux occur with a similar dependency on the pH of the medium (20) but may be considered in the first model to represent two separate effects of ammonia: the first effect to increase K\textsuperscript+/H\textsuperscript+ antiporter activity by increasing the cytoplasmic pH, and the second to displace from the cell surface divalent ions critical to CH\textsubscript{4} synthesis. This model can explain the lack of K\textsuperscript+ efflux from certain bacteria treated with ammonia, if these organisms lack the K\textsuperscript+/H\textsuperscript+ antiporter (20). The second model predicts that displacement of divalent ions from the cytoplasmic membrane by ammonia is the primary event to cause a concomitant efflux of K\textsuperscript+ and loss of methanogenic activity. In support of this model, divalent ions, notably Mg\textsuperscript{2+}, are known to decrease the permeability to K\textsuperscript+ of mitochondrial (32), erythrocyte (33), and yeast (34) membranes. This second model is largely discounted, however, by the finding that in the presence of MgCl\textsubscript{2} ammonia still induced K\textsuperscript+ to efflux, but methanogenic activity was retained (Table I). This finding is consistent with the first model and our previous interpretation of ammonia/K\textsuperscript+ exchange (20), pointing to a second effect of ammonia to displace divalent ions necessary for CH\textsubscript{4} synthesis. Activation by divalent ions shows the site is exposed to the medium, because the ammonia treatment caused little change in the content of cytoplasmic Mg\textsuperscript{2+} or Ca\textsuperscript{2+}. The decline in intracellular ATP concentration (Fig. 2) may explain the loss of methanogenesis, or vice versa, if a tight coupling between CH\textsubscript{4} and ATP synthesis occurs in this methanogen, as it does in Ms. barkeri (18, 30). ATPase inactivation seems unlikely becauseactivation by divalent ions occurs near the cell surface. Activation of a membrane-bound methanogenic enzyme is possible, since in Msp. hungatei part of the methanogenic apparatus may be exposed to the medium (20). Also, the ether-linked membrane lipids (35) could be the activation site. In either event, the importance of the cytoplasmic membrane in methanogenesis from CO\textsubscript{2} is emphasized (4, 29, 30).

Once depleted of K\textsuperscript+ by ammonia treatment, Msp. hungatei cells had lost not only their ability to make CH\textsubscript{4}, but also the ATP pool was diminished and Rb\textsuperscript+ transport was of low activity. Activation of methanogenesis by Mg\textsuperscript{2+} resulted in net ATP synthesis. Under these conditions, Rb\textsuperscript+ was taken up by the K\textsuperscript+ transport system to a steady state concentration approximating the K\textsuperscript+ content of nondepleted cells. Similarly, in starved cells of Ms. barkeri, the onset of methanogenesis upon methanol addition led to ATP synthesis (30). Since acetate is a central intermediate in pathways of intermediary metabolism in methanogens and our culture of Msp. hungatei cannot form acetate from CO\textsubscript{2} (36), it is likely that methanogenesis is linked to ATP synthesis via a chemiosmotic mechanism rather than by substrate phosphorylation. This agrees with most previous predictions (4, 29, 30).

Little net movement of protons is associated with methanogenic activity in K\textsuperscript+-depleted cells in an acidic buffer lacking Na\textsuperscript+ and K\textsuperscript+ (Table II). This is reminiscent of the need for electrogenic K\textsuperscript+ influx to serve as a counterion to allow increased rates of H\textsuperscript+ ejection and establishment of \Delta pH in S. faecalis (37), E. coli (38), or V. alginolyticus (10). In most cases, K\textsuperscript+ influx is associated with increases in the glycolytic (37) or respiratory (10, 39) rates. In contrast, no change in the methanogenic rate is seen in Msp. hungatei during the

Fig. 10. An increase in the osmotic pressure of the medium can influence the cytoplasmic Rb\textsuperscript+ content at steady state. A series of cell suspensions were K\textsuperscript+-depleted and washed twice with prereduced 0.1 M Hepes (pH 6.5) containing 10 mM MgCl\textsubscript{2}. Each cell pellet (3.4 mg, dry weight) was resuspended in 5 ml of prereduced solutions of 0.02 M Hepes, 10 mM MgCl\textsubscript{2}, and glucose from 0 to 0.5 M and transferred by syringe to 60-ml serum bottles containing CO\textsubscript{2}/H\textsubscript{2} (1:4, v/v). After a 30-min incubation, "Rb" was added (5.36 \times 10\textsuperscript{5} cpm/\mu mol), and transport was assayed after 4 and 60 min. Slopes of the methanogenic rate were taken to correspond to the times when transport was measured (curves 1 and 2 correspond to the 4 and 60 min transport points, respectively).
uptake of K⁺. Thus, the present results are consistent with a decreased energy demand for proton efflux to occur (passive proton efflux is not excluded), as a result of electronic K⁺ (or Rb⁺) influx. The possibility of an ATPase exchanging H⁺/K⁺ as hypothesized for E. coli (40) should be considered, but a 1:1 stoichiometry is excluded by the systemic nature of K⁺ uptake.

Blaut and Gottschalk (30) suggested that methyl coenzyme M reduction is linked to proton translocation in Ms. barkeri metabolizing methanol, based on the ability of an uncoupler to relieve the inhibition to CH₃ synthesis caused by N₅N'-dicyclohexylcarbodiimide. In M. trichosporium OB3b a net proton influx during methanogenesis in alkaline media may be interpreted in favor of a model based on internal vesicles as hypothesized for E. coli. However, in another eubacterium the pmf formation in methanogens is beyond the scope of this study and remains unresolved. However, interpretations based on internal membranes are excluded by the ability of an uncoupler to relieve the inhibition to CH₃ synthesis caused by an ATPase exchanging H⁺/Na⁺ in acidic media. K⁺ influx is linked to the efflux of H⁺ or Na⁺ in certain eubactera (9, 22). Also, K⁺ uptake in Msp. hungatei is electrogenic and associated with the formation of a ΔpH in acidic media. K⁺ influx is linked to the influx of H⁺ or Na⁺ in several eubacteria (10, 37, 38). A role for cytoplasmic K⁺ as an osmoregulator, as in E. coli (5), is implied from the increase in cytoplasmic K⁺ concentrations at steady state when the osmotic pressure of the medium is increased (Fig. 10). In Mycoplasma mycoides, Na⁺-dependent K⁺ influx may be catalyzed by a Na⁺/K⁺-ATPase, typical of higher organisms (44). However, in another eubacterium (13) and Msp. hungatei, K⁺ transport does not depend on Na⁺ movements.

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