*Biochimica et Biophysica Acta*, 725 (1983) 280–288
Elsevier

BBA 41402

# MEASUREMENT AND SIGNIFICANCE OF THE MEMBRANE POTENTIAL IN *METHANOBACTERIUM BRYANTII* *

KEN F. JARRELL and G. DENNIS SPROTT

*Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6 (Canada)*

(Received June 2nd, 1983)

*Key words: Membrane potential; Methanogenesis; (Methanobacterium bryantii)*

**The membrane potential ($\Delta\psi$) of *Methanobacterium bryantii* was 133–142 mV as measured from the distribution of $^{86}Rb^+$ in valinomycin-treated cells, and was considerably higher than that obtained using triphenylmethylphosphonium in the presence of tetraphenylboron. The $\Delta\psi$ measured using the $Rb^+$/valinomycin method was sensitive to certain ionophores including gramicidin, nigericin, carbonyl cyanide *m*-chlorophenylhydrazone and 3,3′,4′,5-tetrachlorosalicylanilide. It was also dissipated by 1 mM tetraphenylphosphonium and was abolished in heat-treated or permeabilized cells. The $\Delta\psi$ could be varied by adjusting the extracellular potassium concentration in valinomycin-treated cells. Monensin-treated cells possessed a significantly increased $\Delta\psi$, as monitored by the $Rb^+$/valinomycin method. Tetraphenylphosphonium cation (1 mM) abolished methane synthesis, intracellular ATP and $\Delta\psi$, supporting a role for $\Delta\psi$ in ATP and $CH_4$ synthesis. However, lower concentrations of the lipophilic cation (50 $\mu$M) greatly elevated both the intracellular ATP concentration and $\Delta\psi$ but decreased the rate of $CH_4$ synthesis by almost 50%. Thus, tetraphenylphosphonium cation exerts a primary inhibitory effect on $CH_4$ synthesis which cannot be attributed to the loss of $\Delta\psi$ or ATP.**

## Introduction

Unlike many bacteria which generate a transmembrane pH gradient ($\Delta$pH, inside alkaline) of considerable magnitude, the methanogens examined thus far, including *Methanospirillum hungatei* [1], *Methanobacterium thermoautotrophicum* [1,2] and *Methanobacterium bryantii* [3], all possess an internal pH of approx. 6.7 at neutral external pH. These results were obtained from distributions of permeant weak acids and bases [4] using either centrifugation [1] or flow dialysis [2] methods. Hence at growth pH, little if any $\Delta$pH is maintained and its contribution to the proton-motive force can be negligible. This, of course, does not rule out the possible presence of localized pH gradients within the cytoplasmic membrane [5], since such gradients would not be measured by these techniques but could be available for important physiological processes.

It does appear that the proton-motive force of the methanogens examined consists predominantly of the membrane potential ($\Delta\psi$, inside negative) and thus the measurement of $\Delta\psi$ becomes very important in understanding the bioenergetics of these archaebacteria [6]. It is not known how methanogens generate $\Delta\psi$; in fact very few studies on $\Delta\psi$ have been reported. Doddema et al. [7] qualitatively measured $\Delta\psi$ with fluorescent dyes in

* NRCC paper No. 22672.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TPB, tetraphenylboron; TPMP, triphenylmethylphosphonium; TCS, 3,3′,4′,5-tetrachlorosalicylanilide; TPP, tetraphenylphosphonium; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

both cells and vesicles of *M. thermoautotrophicum*. The measured $\Delta\psi$ was dissipated with very high concentrations of nigericin (0.4 mM) but not gramicidin, and anaerobic conditions were not prerequisite. Also, using *M. thermoautotrophicum*, Sauer et al. failed to detect a $\Delta\psi$ either inside positive [2] or inside negative [8] using the distributions of $SCN^-$ or $TPMP^+$, respectively. Kell et al. [9] reported the uptake of dibenzyldimethylammonium cation into membrane vesicles of *M. thermoautotrophicum*, indicating the formation of the $\Delta\psi$ (inside negative) although the magnitude was not reported. A $\Delta\psi$ (inside negative) of 130–173 mV, completely sensitive to aeration, was measured in *M. thermoautotrophicum* cells using a $TPP^+$-sensitive electrode [48].

Some of these discrepancies may be attributed to the different techniques used in attempts to measure $\Delta\psi$. For example, optimal uptake of $TPMP^+$ or $TPP^+$ often requires the presence of small quantities of a counterion like $TPB^-$ [10] or phenyldicarbaundecaborane ($PCB^-$ [11]). We have previously measured the $\Delta\psi$ in both *M. hungatei* cells and spheroplasts using $TPMP^+$ in the presence of $TPB^-$ and found a $\Delta\psi$ of 74–79 mV in cells [1,12] and 103 mV in spheroplasts [12]. Attempts to measure $\Delta\psi$ using $^{86}Rb^+$ accumulation in the presence of valinomycin were unsuccessful in this organism. On the other hand, $\Delta\psi$ could be measured with both the $^{86}Rb$/valinomycin and the $TPMP^+/TPB^-$ systems in *M. thermoautotrophicum* with $\Delta\psi$ values of 133 and 119 mV, respectively, being recorded [1]. Comparisons of these two methods in other bacteria indicated a close similarity in some cases [13–15] and major discrepancies in other studies [11,16–18].

Recently, in a study on the effects of ionophores and metabolic inhibitors on various bioenergetic properties of *M. bryantii* ($CH_4$ synthesis, intracellular ATP and potassium levels, $\Delta$pH and $\Delta\psi$) we determined $\Delta\psi$ to be 127 mV using the $TPMP^+/TPB^-$ system [3]. This $\Delta\psi$ was, however, only partially dissipated using protonophores and indeed there is very little in the literature on the sensitivity of $\Delta\psi$ in methanogens to inhibitors. We have expanded that incipient study of $\Delta\psi$ in *M. bryantii* in this report by comparing $\Delta\psi$ measured by both $^{86}Rb$/valinomycin and $TPMP^+/TPB^-$ methods. We now show that the $\Delta\psi$ measured using the $^{86}Rb^+$/valinomycin method is much higher than that determined from $TPMP^+$ distribution and, unlike the case with $TPMP^+$, the $\Delta\psi$ measured with the $^{86}Rb^+$/valinomycin method can be totally dissipated by certain ionophores.

## Materials and Methods

*Organism and growth conditions.* *M. bryantii* MOH [19] was obtained from M.P. Bryant. Cultures were maintained at 35°C by weekly transfers in a prereduced carbonate-buffered synthetic medium (S medium [20]) under an atmosphere of $H_2/CO_2$ (4 : 1, v/v). Cultures were shaken at 150 rpm and the bottles were pressurized daily with $H_2/CO_2$ (4 : 1, v/v) to 170 kPa.

*Reagents.* [$^3$H]$TPMP^+$ (bromide salt) and $^{86}RbCl$ were obtained from New England Nuclear, Canada. Valinomycin, gramicidin, $TPB^-$ and CCCP were purchased from Sigma. Nigericin was obtained from Calbiochem-Behring Corp., $TCS^-$ from Fisher Scientific Co. and $TPP^+$ (chloride salt) from ICN Pharmaceuticals, Inc. Monensin was a kind gift of J. Kwong (Eli-Lilly Canada, Inc.). Acetylene (99.5% purity) was received from Union Carbide Canada, Ltd.

*General anaerobic techniques.* Methods for monitoring and maintaining anaerobiosis, anaerobic centrifugation with modified Corex centrifugation tubes to concentrate cells, and preparation of anaerobic buffers have all been described previously [1]. Potassium-free S medium was prepared by substituting sodium phosphate for potassium phosphate in mineral solutions 1 and 2 [20]. In some experiments involving CCCP, buffers or potassium-free S medium were reduced with $H_2S$ rather than cysteine/sodium sulfide.

*Internal water content.* In all experiments, an internal space of 2.08 $\mu$l/mg dry weight was used to calculate in/out values [21].

*Intracellular ATP concentration.* ATP was measured by liquid scintillation spectrometry using luciferin-luciferase (Sigma) as described in detail elsewhere [22,23].

*Methane synthesis.* Methane synthesis was followed by assaying the headspace gas by gas-liquid chromatography using previously described conditions [24].

*Measurement of* $\Delta\psi$. Anaerobic pellets from 20

ml of a culture in exponential phase were resuspended in 5 ml of $K^+$-free S medium or buffer, transferred to 60-ml serum bottles under $H_2/CO_2$ (4:1, v/v) and incubated with shaking at 35°C. Valinomycin was added to a final concentration of 20 μM 15 min prior to the addition of 100 μM $^{86}RbCl$. Aliquots (0.5 ml) were removed at timed intervals, filtered (HA filters, 0.45 μm, Millipore Corp.) and washed with 5 ml of 0.1 M LiCl. Filters were placed in 5 ml water and counted on the tritium channel of a liquid scintillation counter. Varying the final $Rb^+$ concentration between 10 and 500 μM did not change the gradient ($[Rb^+]_{in}/[Rb^+]_{out}$) at steady state. In experiments measuring $\Delta\psi$ with $TPMP^+$, [$^3$H]$TPMP^+$ was added to 10 μM and a 0.5 ml sample filtered and washed immediately as a measure of nonspecific binding. $TPB^-$ (2 μM) was then added and subsequent filtrations performed. For $TPMP^+$ usage, filters were presoaked in unlabeled 10 μM $TPMP^+$ to reduce binding of the label [25]. Filters with $TPMP^+$ were counted after overnight solubilization in Aquasol (New England Nuclear, Canada). The zero-time control values were very similar to those obtained by filtering $TPMP^+$ in the absence of cells, indicating very little nonspecific binding of the label to cells.

Rubidium uptake in the presence and absence of valinomycin was also examined using a centrifugation assay with modified Corex centrifuge tubes. Cells, in $K^+$-free S medium, were left in the anaerobic centrifuge tubes, treated with valinomycin and $^{86}RbCl$ as above and the cells centrifuged (10000 × *g* for 10 min) after 30 min. The intracellular $^{86}Rb^+$ accumulated was determined after correction for extracellular $^{86}Rb^+$ trapped in the pellet [1].

The $\Delta\psi$ was calculated from the Nernst equation, $\Delta\psi = -RT/ZF \ln[\text{cation}]_{in}/[\text{cation}]_{out}$ where at 35°C, $\Delta\psi = 61.1 \log[\text{cation}]_{in}/[\text{cation}]_{out}$ [26].

*Effect of headspace gas on* $\Delta\psi$. Anaerobic pellets from 20 ml of cells were resuspended in 5 ml of anaerobic 100 mM Hepes buffer (pH 6.5). The concentrated cells were transferred to 60-ml serum bottles containing the desired atmosphere and flushed thoroughly with the desired gas. The bottles were sealed and incubated with shaking at 35°C for 90 min before $\Delta\psi$ was determined. These experiments were performed in 100 mM Hepes buffer instead of S medium because the buffer was found to be much better in maintaining a relatively constant pH under the different atmospheres.

*Effect of external potassium on* $\Delta\psi$. Cells, centrifuged anaerobically, were resuspended in reduced $K^+$-free S medium to which KCl was added to give final concentrations ranging from 0.1 to 100 mM. Following transfer of the cells to 60-ml serum bottles under $CO_2/H_2$, valinomycin was added and the cells shaken at 35°C. 15 min later $^{86}Rb^+$ (100 μM) was added and accumulation of the label was followed by the filtration method described above. The actual concentrations of $K^+$ in the $K^+$-free S medium and 100 mM Hepes buffer used in these studies were 0.38 and 0.11 mM, respectively, as determined by atomic absorption spectroscopy.

*Treatment of cells with inhibitors.* The reagents were stored as concentrated stock solutions in ethanol under $H_2/CO_2$ (4:1, v/v). The inhibitors were added for 10 min prior to the start of the $\Delta\psi$ determination except for acetylene which was present for 2 h and for which a separate control was run. The final concentrations of inhibitors used are listed in Table I. Heat-treated cells were in-

TABLE I

EFFECT OF INHIBITORS ON $\Delta\psi$ IN *METHANOBACTERIUM BRYANTII*

Measurements of $\Delta\psi$ was performed in $K^+$-free S medium after incubation of cells with inhibitors for 10 min ($C_2H_2$, 2 h).

| Inhibitor | $[Rb^+]_{in}/[Rb^+]_{out}$ | $\Delta\psi$ (mV) |
|---|---|---|
| None (control) | 173 | 137 |
| Gramicidin (10 μg/ml) | 0 [b] | 0 |
| TCS (20 μM) | 42 | 99 |
| Monensin (20 μM) | 262 | 148 |
| Nigericin (20 μM) | 47 | 102 |
| TPP (1 mM) | 36 | 95 |
| *N*-Ethylmaleimide (1 mM) | 160 | 135 |
| $C_2H_2$ (290 μM) | 116 | 126 |
| *n*-butanol (5%, v/v) | 0 [b] | 0 |
| CCCP (20 μM) | 161 | 135 |
| Control ($H_2S$) [a] | 144 | 132 |
| CCCP ($H_2S$) [a] | 31 | 91 |

[a] Measurement of $\Delta\psi$ in $K^+$-free S medium reduced with $H_2S$ instead of cysteine-$Na_2S$.

[b] No uptake of $^{86}Rb^+$ above the blank value.

cubated at 90–100°C for 10 min. Cells permeabilized with *n*-butanol received 5% (v/v) *n*-butanol previously stored anaerobically under an atmosphere of $H_2/CO_2$ (4:1, v/v).

*Effect of $TPP^+$ on intracellular ATP concentration, methanogenesis and $\Delta\psi$.* Cells were concentrated 3-fold by anaerobic centrifugation and transferred to 60-ml serum bottles under $H_2/CO_2$ (4:1, v/v). $TPP^+Cl^-$ was added, from concentrated stock solutions, to a final concentration of 10 $\mu$M–1 mM and the cells incubated with shaking at 35°C. Headspace gas was analyzed for methane as described above over a 2 h incubation period. At the end of the 2 h period, an aliquot of cells was removed for ATP determination or the $\Delta\psi$ was determined using either the valinomycin/$^{86}Rb^+$ or the $TPMP^+/TPB^-$ method as described above. To examine the kinetics of the $TPP^+$ effect on ATP levels, samples of cells, incubated in the presence of $TPP^+$, were removed for ATP determination at 30 min intervals over a 3 h incubation period.

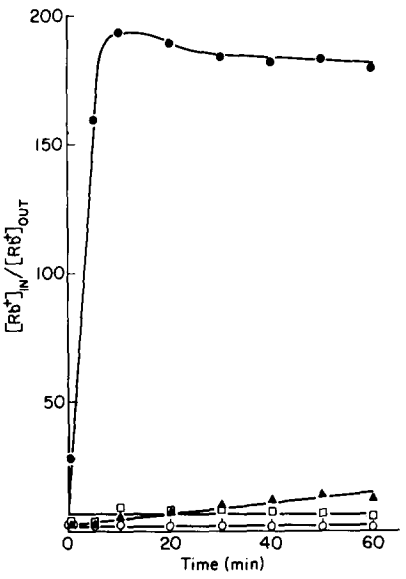


Fig. 1. Kinetics of $^{86}Rb^+$ accumulation by *M. bryantii*. Cells were suspended in $K^+$-free S medium and $^{86}Rb^+$ accumulation was followed in cells which were (●) or were not (▲) pretreated for 15 min with valinomycin. Some of the valinomycin-treated cells were previously heat treated (○) or permeabilized with 5% (v/v) butanol (□).

## Results

*Measurement of $\Delta\psi$ using $^{86}Rb^+$ distribution in valinomycin-treated cells*

In the absence of valinomycin, *M. bryantii* very slowly accumulated $^{86}Rb^+$. The ionophore, which allows for distribution of $Rb^+$ in accordance with the magnitude of $\Delta\psi$ [4], caused a very marked accumulation of $^{86}Rb^+$ (Fig. 1). A steady-state distribution was reached within 10 min, similar to the uptake of the ion in valinomycin-treated *Escherichia coli* [13]. The final $[Rb^+]_{in}/[Rb^+]_{out}$ values varied with different batches of cells between 150 and 210, indicating a $\Delta\psi$ of 133–142 mV (inside negative). $^{86}Rb^+$ accumulation was eliminated if the cells were subjected to a heat treatment (95°C, 10 min) or permeabilized with 5% (v/v) *n*-butanol as reported for *E. coli* [11].

We compared the $^{86}Rb^+$ distribution in valinomycin-treated cells obtained by either the filtration or centrifugation assay and found the values to be almost identical. In one representative experiment $[Rb^+]_{in}/[Rb^+]_{out}$ was 185 by filtration and 213 by centrifugation, indicating $\Delta\psi$ values of 139 and 142 mV, respectively. This demonstrated that there was little loss of accumulated $^{86}Rb^+$ during the aerobic washing procedure used in the filtration assay, thus differing from reports in *E. coli* where the filtration method yielded $\Delta\psi$ measurements 10–20 mV lower than a centrifugation assay [14].

*Sensitivity to ionophores of $^{86}Rb^+$ uptake in valinomycin-treated cells*

The accumulation of $^{86}Rb^+$ in valinomycin-treated cells was examined after pretreatment of the cells with various agents (Table I). Gramicidin, a rather nonspecific ionophore which allows passage of $Na^+$, $K^+$ and $H^+$ [27], was the most effective agent, totally eliminating $^{86}Rb^+$ uptake. TCS, a protonophore [28], was very effective in reducing $^{86}Rb^+$ accumulation as were $TPP^+$, a permeant organic cation expected to dampen $\Delta\psi$ when at high concentration, and nigericin which mediates a $K^+$-$H^+$ exchange [28]. Monensin, an $Na^+$-$H^+$ exchanger [28] which can also cause massive $K^+$ efflux in *M. bryantii* [3] increased $\Delta\psi$ while $C_2H_2$, which is a potent growth-inhibitory agent for methanogens [22] and an agent which can dissipate imposed pH gradients (inside alkaline

[3,22]), caused a partial decline in $\Delta\psi$. The sulfhydryl reagent *N*-ethylmaleimide had no effect on $\Delta\psi$, indicating that $^{86}Rb^+$ uptake was in all probability not mediated by carrier proteins (i.e., $^{86}Rb^+$ was not being transported by a $K^+$ transport system to any significant extent). The protonophore CCCP had no effect on $\Delta\psi$ in cysteine-sulfide-reduced media but it was very effective in media reduced with $H_2S$. This is due to an interaction of CCCP with cysteine [29] as observed previously [3].

Treatment with ionophores of cells which had already accumulated $^{86}Rb^+$ caused a dramatic efflux of the label (Fig. 2), indicating the lack of significant binding of the $^{86}Rb^+$ to internal cell components. This, combined with the low blanks observed with heat-treated or permealized cells, which indicated low binding of label to external wall and membrane sites, means irreversible binding of $^{86}Rb^+$ to cell components is not an important source of error in these experiments.

*$\Delta\psi$ as a function of extracellular KCl concentration*

The effect of KCl addition to the external medium on $\Delta\psi$ measured from $^{86}Rb^+$ distribution in valinomycin-treated cells is depicted in Fig. 3. An addition of 0.1 mM KCl above the 0.38 mM $K^+$ present in 'K-free' S medium had no effect on $\Delta\psi$ but 1 mM KCl started to dampen $\Delta\psi$. By 10 mM KCl, about 75% of the $^{86}Rb^+$ accumulation was prevented with total inhibition being observed at 100 mM KCl. At each KCl concentration tested, a new steady-state distribution of $^{86}Rb^+$ was obtained by 10 min. This indicates that $K^+$, at the concentration tested, did not saturate the valinomycin channels and thus did not invalidate $\Delta\psi$ measurements using $^{86}Rb^+$. Thus, as in other bacteria [30], it is possible in *M. bryantii* to vary the $\Delta\psi$ to a desired level by changing the ratio of internal to external potassium in valinomycin-treated cells.

*Effect of headspace gas on $\Delta\psi$*

$\Delta\psi$ was maximal only under an atmosphere of $CO_2/H_2$ (Table II). All other headspaces tested, including air, resulted in a 40% reduction in $^{86}Rb^+$

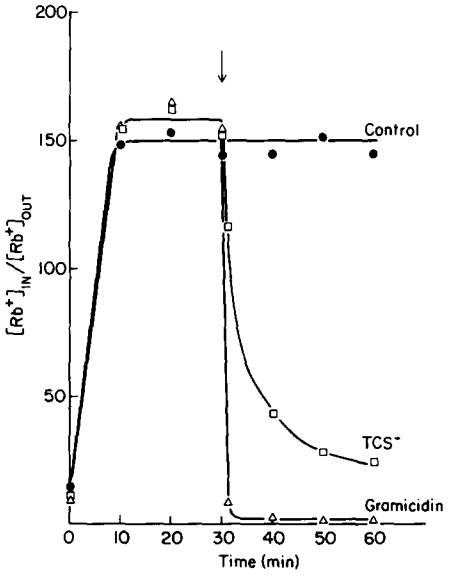


Fig. 2. Effect of ionophores on release of accumulated $^{86}Rb^+$. Cells suspended in $K^+$-free S medium were treated for 15 min with valinomycin prior to $^{86}Rb^+$ addition. Accumulation was allowed to proceed to steady state before the addition (at the arrow) of ethanol (control, (●)), TCS (□) or gramicidin (△). Retention of the label was followed with the filter assay.

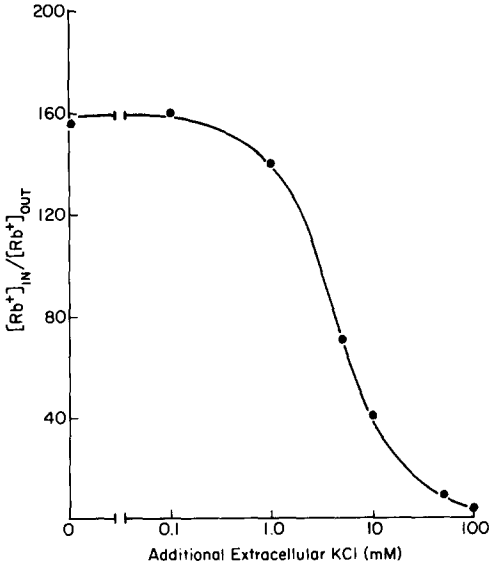


Fig. 3. The membrane potential, measured from $^{86}Rb^+$ distribution, of valinomycin-treated *M. bryantii* cells as a function of extracellular KCl concentration. Cells, suspended in $K^+$-free S medium to which was added the indicated KCl concentration, were pretreated with valinomycin and $\Delta\psi$ measured as the $^{86}Rb^+$ distribution. $K^+$-free S medium contained 0.38 mM $K^+$ as determined by atomic absorption spectroscopy.

TABLE II

INFLUENCE OF HEADSPACE GAS ON THE $\Delta\psi$ IN *METHANOBACTERIUM BRYANTII*

The cells were resuspended in $K^+$-free S medium or in 100 mM Hepes buffer adjusted with NaOH. The pH values at the end of the experiment were: S medium, 6.61; Hepes buffer, $CO_2/H_2$ 6.50; $CO_2/N_2$, 6.51; $N_2$, 6.87; $H_2$, 6.94; air, 6.86. $[Rb^+]_{in}/[Rb^+]_{out}$ was measured at steady state.

| Headspace | $[Rb^+]_{in}/[Rb^+]_{out}$ | $\Delta\psi$ (mV) |
|---|---|---|
| S medium, $CO_2/H_2$ | 197 | 140 |
| Hepes buffer, $CO_2/H_2$ | 559 | 168 |
| Hepes buffer, $CO_2/N_2$ | 321 | 153 |
| Hepes buffer, $N_2$ | 309 | 152 |
| Hepes buffer, $H_2$ | 363 | 156 |
| Hepes buffer, air | 326 | 154 |

uptake. These experiments were performed on cells suspended in anaerobic 100 mM Hepes buffer (adjusted with NaOH to pH 6.5), since the pH of this solution was relatively constant under the different headspaces. Although the pH of S medium was much more dependent on headspace gas, we did observe the same trends in $\Delta\psi$ regardless of whether the cells were suspended in medium of Hepes buffer.

Why the $^{86}Rb^+$ accumulation is so much higher in Hepes buffer compared to S medium remains to be explained but may be caused by the differing ionic contents. The Hepes buffer contained less $K^+$ than the $K^+$-free S medium (0.1 mM compared to 0.38 mM, respectively), but addition of sufficient KCl to the Hepes buffer to bring the concentration to 1 mM did not reduce the $\Delta\psi$ to S medium levels. Importantly, uptake of $^{86}Rb^+$ by cells suspended in Hepes buffer was prevented by a prior heat treatment or by permeabilizing the cells with *n*-butanol. In contrast to these results, in the $TPMP^+$ assay $\Delta\psi$ was not significantly different in the two suspending solutions (data not shown). We can only state that the $\Delta\psi$ measured in S medium must be taken as a minimum value.

*Comparison of $\Delta\psi$ measured with $^{86}Rb^+$/valinomycin and $TPMP^+/TPB^-$*

$^{86}Rb^+$ distribution in valinomycin-treated cells and the distribution of permeant organic cations like $TPP^+$ and $TPMP^+$ are two of the most common methods for monitoring $\Delta\psi$. We tested both methods on *M. bryantii*. Since the $^{86}Rb^+$ method necessarily involves valinomycin-treated cells we followed $TPMP^+$ accumulation (in the presence and absence of the counterion $TPB^-$) in both control cells and in cells treated with valinomycin. It is very obvious that $^{86}Rb^+$ distribution led to a much higher estimation of $\Delta\psi$ than did $TPMP^+$ (Table III). The presence of $TPB^-$ was needed for maximum uptake of $TPMP^+$ both in

TABLE III

COMPARISON OF $Rb^+$ AND $TPMP^+$ DISTRIBUTION AS A MEASURE OF $\Delta\psi$ IN *METHANOBACTERIUM BRYANTII*

| Cation | Valinomycin pretreatment | $[Cation]_{in}/[Cation]_{out}$ | $\Delta\psi$ (mV) |
|---|---|---|---|
| $Rb^+$ | yes | 148 | 133 |
| $Rb^+$ | no | 4 | 40 |
| $TPMP^+$ (with $TPB^-$) | yes | 91 | 120 |
| $TPMP^+$ (without $TPB^-$) | yes | 50 | 104 |
| $TPMP^+$ (with $TPB^-$) | no | 64 | 110 |
| $TPMP^+$ (without $TPB^-$) | no | 37 | 96 |

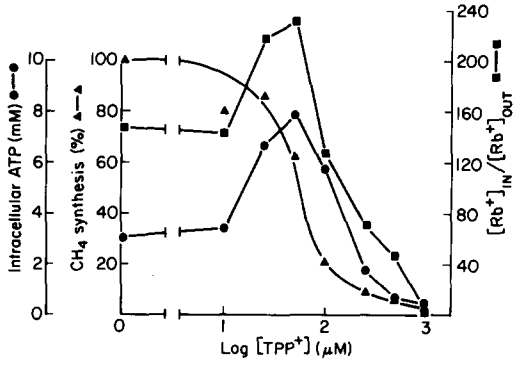


Fig. 4. Effects of extracellular $TPP^+$ concentration on intracellular ATP concentration, methane synthesis and $\Delta\psi$. Cells were suspended in $K^+$-free S medium to which was added the indicated level of $TPP^+$. The rate of $CH_4$ synthesis (▲) was determined by following the accumulation of $CH_4$ in the culture headspace over a 2 h period. After 2 h, samples of cells were removed for determination of ATP levels and for $\Delta\psi$ measurement using the distribution of $^{86}Rb^+$ after pretreatment of the cells with valinomycin. The rate of methane synthesis in the control was 43 μmol $CH_4$/h per mg dry weight. $CH_4$ synthesis was linear over the 2 h incubation period.

valinomycin-treated and control cells, with valinomycin-treated cells showing a larger $\Delta\psi$ than cells not so pretreated.

*Effect of $TPP^+$ on methanogenesis, intracellular ATP and $\Delta\psi$*

High concentrations of $TPMP^+$ (1–2 mM) have been shown previously to inhibit methanogenesis in *M. bryantii* [3] and in cell extracts of *M. ruminantium* [31]. $TPP^+$ was more potent than $TPMP^+$ in *M. bryantii* with 50% inhibition of $CH_4$ synthesis occurring at 60 $\mu$M $TPP^+$ (Fig. 4) compared to 230 $\mu$M $TPMP^+$ [3]. At high levels (1 mM), $TPP^+$ caused almost a total dissipation of $CH_4$ synthesis, ATP content and $\Delta\psi$ (note that the lower $[Rb^+]_{in}/[Rb^+]_{out}$ ratio when compared with Table I may be explained by the different times of exposure to $TPP^+$ used prior to measuring $^{86}Rb^+$ uptake). However, at lower concentrations, especially 50 $\mu$M, $TPP^+$ caused marked increases in both intracellular ATP content and $\Delta\psi$ (measured with either $^{86}Rb^+$/valinomycin (Fig. 4) or $TPMP^+/TPB^-$ (not shown)) while decreasing $CH_4$ synthesis by nearly 50%. When examined at various time intervals after exposure of cells to 0, 50 or 1000 $\mu$M $TPP^+$ (Fig. 5) the intracellular ATP content was relatively constant in the absence of

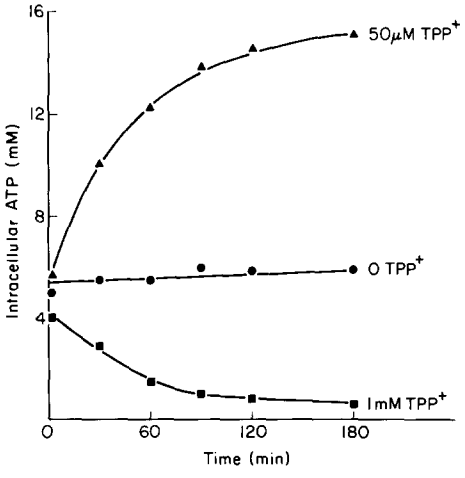


Fig. 5. Effect of extracellular $TPP^+$ concentration on the intracellular ATP concentration of *M. bryantii*. $TPP^+$ was added, to a final concentration of 0, 50 $\mu$M or 1 mM, to cells suspended in S medium. At the times indicated aliquots were removed and the ATP content determined using the luciferin-luciferase assay.

$TPP^+$ over a 3 h period; cells exposed to 1 mM $TPP^+$ showed a gradual drop in ATP over the initial 1 h exposure period and thereafter a low level of ATP remained. In contrast, cells incubated in the presence of 50 $\mu$M $TPP^+$ showed a continuous increase in their intracellular ATP concentration for about 2 h before steady state was achieved. The new ATP concentration represented an approx. 2.5-fold increase.

## Discussion

In the few studies conducted on $\Delta\psi$ in methanogens organic cations have usually been employed [1–3,8,9]. Here we report the magnitude of $\Delta\psi$ to be much higher according to distributions of $^{86}Rb^+$ (in the presence of valinomycin) as compared to $TPMP^+$ (in the presence of $TPB^-$). In agreement with a recent report in *E. coli* [11] we feel that the $\Delta\psi$ measured from $^{86}Rb^+$ distribution is the closer of the two methods to the correct value. Several points support this view. First, the uptake of $^{86}Rb^+$ required valinomycin. Thus, the little $^{86}Rb^+$ that could have entered through a carrier-mediated naturally occurring transport system was swamped by the distribution that occurred in the presence of valinomycin. Second, there was very little nonspecific irreversible binding of $^{86}Rb^+$ either to external cell structures or to intracellular components as indicated by the low blanks obtained with heat-treated or permeabilized cells and by the total efflux of accumulated label upon gramicidin treatment. This contrasts to the well described binding problems possible with the organic cations [14,30,32,33]. These binding problems can occur with *M. bryantii* also, presumably to internal cell components, since heat or butanol treatment often give erroneously high blanks in the $TPMP^+$ system. Finally, further problems arise in our system with $TPMP^+$ because the uptake of the label is only poorly inhibited by those ionophores which give much greater inhibitions of $^{86}Rb^+$ uptake (this study, and [3]).

The $\Delta\psi$ measured with $^{86}Rb^+$/valinomycin in *M. bryantii* exhibited characteristics typical of other bacteria. It was sensitive to protonophores, heat treatment and permeabilization of the cells and thus was clearly not due to a Donnan potential. It was dissipated gradually in valinomycin-treated

cells as the external $K^+$ concentration was raised, allowing one to manipulate the magnitude of $\Delta\psi$. An intriguing finding was the relative insensitivity of $\Delta\psi$ of these obligate anaerobes to $O_2$. Doddema et al. [7] observed in a qualitative way that anaerobic conditions were not prerequisite to measurement of $\Delta\psi$ in *M. thermoautotrophicum*, although others have reported, using different methods of measurement, total sensitivity of $\Delta\psi$ of *M. thermoautotrophicum* to $O_2$ [48]. In *M. bryantii* we found that approx. 60% of the $Rb^+$ accumulation remained during exposure of the cells to air. This would appear to put some constraints on how $\Delta\psi$ is generated and maintained in this organism. One obvious possibility is $H^+$ pumping by hydrogenase [34,35], yet this enzyme has been reported by several laboratories using different methanogens to be oxygen sensitive [36–38]. The lack of involvement of hydrogenase in generating a transmembrane $\Delta\psi$ is consistent with the findings of Spencer et al. [39]. They reported the intracellular production of $H^+$, which suggested to them that either hydrogenase was not involved in production of a chemiosmotic proton gradient or, if so, the gradient was between the cytosol and the frequently observed but possibly artefactual [3] internal vesicles. Hydrolysis of ATP to establish $\Delta\psi$ seems unlikely because intracellular ATP concentrations decline dramatically upon exposure of methanogens to $O_2$ [23,40]. Moreover, $C_2H_2$ has only a minor effect on $\Delta\psi$ but totally abolishes the ATP content of *M. bryantii* [22]. Another method of generating $\Delta\psi$, as suggested by Perski et al. [41] is through an electrogenic $Na^+$ pump. Coupling to an $Na^+/H^+$ antiporter could convert the sodium-motive force to a proton-motive force as required to explain the sensitivity of methanogenesis to protonophores (reviewed in Ref. 42). No direct evidence exists yet for such an antiporter in methanogens. Further, the proposed mechanism must function to give $\Delta\psi$ with little or no pH gradient at neutral external pH, which implies that proton efflux is the primary event with $Na^+/H^+$ antiport as a possible secondary transporter. To make the issue more complex, differences in ion movements among the methanogens must exist, since the $Na^+$ gradient was of opposite orientation in *M. thermoautotrophicum* (outwardly directed) and *M. hungatei* (inwardly directed [43]). The $Na^+$ gradient has not been determined for *M. bryantii*.

Although methanogens possess a number of $O_2$-insensitive enzymes [44,45] and protein synthesis in a cell-free system was not $O_2$ sensitive [46] it was still unexpected to discover $\Delta\psi$ to be relatively $O_2$ stable. It would certainly be to the cell's advantage to maintain an energy reserve after brief exposures to $O_2$ in its natural habitats, especially since methanogens lack a $\Delta$pH in medium of pH 6.6–6.8 [1,3] and intracellular ATP is depleted upon $O_2$ exposure [23,40]. It is now realized that, contrary to early beliefs, methanogens can survive exposures to $O_2$ (Refs. 44 and 45; and Patel, G.B., personal communication), although growth and methane synthesis only occur under strictly anaerobic conditions.

We have found, as reported earlier [3,31], that lipophilic cations at millimolar concentrations are potent inhibitors of methane synthesis. These concentrations strongly inhibited $\Delta\psi$ and resulted in lowered amounts of cellular ATP (Fig. 4). However, at 50 $\mu$M $TPP^+$, both $\Delta\psi$ and intracellular ATP concentrations were increased, yet methanogenesis was still inhibited almost 50%. $TPP^+$ appears to exert an effect on some step in methanogenesis other than at a putative ATP- or $\Delta\psi$-requirement step, possibly interfering directly or indirectly with some essential enzyme or cofactor.

The $^{86}Rb^+$/valinomycin system to measure $\Delta\psi$ appears satisfactory for both *M. thermoautotrophicum* [1] and *M. bryantii*, although the kinetics of uptake are quite different in the two organisms. This system was not effective for *Mm. hungatei* cells [1]. However, since *Mm. hungatei* cells were not sensitive to inhibition of $CH_4$ synthesis by valinomycin while spheroplasts obtained from these cells were [12], it appears the problem may be due to poor penetrability of valinomycin through the sheath or inner wall. The usefulness of valinomycin to estimate $\Delta\psi$ in other methanogens has yet to be tested. Our success with *M. bryantii* may be attributed, in some measure, to the large lesions occurring in the cell wall following growth in medium containing limiting amounts of nickel and ammonium [24]. This may have an effect equivalent to the EDTA treatment required to sensitize *E. coli* to valinomycin [47].

## References


1 Jarrell, K.F. and Sprott, G.D. (1981) Can. J. Microbiol. 27, 720–728
2 Sauer, F.D., Erfle, J.D. and Mahadevan, S. (1981) J. Biol. Chem. 256, 9843–9848
3 Jarrell, K.F. and Sprott, G.D. (1983) Arch. Biochem. Biophys. 225, 33–41
4 Rottenberg, H. (1979) Methods Enzymol. 55, 547–569
5 Williams, R.J.P. (1982) FEBS Lett. 146, 1–4
6 Woese, C.R., Magrum, L.J. and Fox, G.E. (1978) J. Mol. Evol. 11, 245–252
7 Doddema, H.J., Van der Drift, C., Vogels, G.D. and Veenhuis, M. (1979) J. Bacteriol. 140, 1081–1089
8 Sauer, F.D., Mahadevan, S. and Erfle, J.D. (1980) Biochem. Biophys. Res. Commun. 95, 715–721
9 Kell, D.B., Doddema, H.J., Morris, J.G. and Vogels, G.D. (1981) in Proceedings of the 3rd International Symposium on Microbial Growth on C-1 Compounds (Dalton, H., ed.), pp. 159–170, Heyden & Sons, London
10 Kashket, E.R. (1980) J. Biol. Chem. 254, 8129–8131
11 Bakker, E.P. (1982) Biochim. Biophys. Acta 681, 474–483
12 Sprott, G.D. and Jarrell, K.F. (1983) in 8th College of Biological Sciences Colloquium on Microbial Chemoautotrophy (Strohl, W.R. and Tuovinen, O.H., eds.), Ohio State University Press, to be published March, 1984
13 Ahmed, S. and Booth, I.R. (1981) Biochem. J. 200, 573–581
14 Ghazi, A., Schechter, E., Letellier, L. and Labedan, B. (1981) FEBS Lett. 125, 197–200
15 Zaritsky, A., Kihara, M. and MacNab, R.M. (1981) J. Membrane Biol. 63, 215–231
16 Harold, F.M. and Papineau, D. (1972) J. Membrane Biol. 8, 45–62
17 Guffanti, A.A., Blumenfeld, H. and Krulwich, T.A. (1981) J. Biol. Chem. 256, 8416-8421
18 Bakker, E.P. and Harold, F.M. (1980) J. Biol. Chem. 255, 433–440
19 Bryant, M.P., Wolin, A., Wolin, M.J. and Wolfe, R.S. (1967) Arch. Microbiol. 59, 20–31
20 Breuil, C. and Patel, G.B. (1980) Can. J. Microbiol. 26, 577–582
21 Jarrell, K.F. and Sprott, G.D. (1982) J. Bacteriol. 151, 1195–1203
22 Sprott, G.D., Jarrell, K.F., Shaw, K.M. and Knowles, R. (1982) J. Gen. Microbiol. 128, 2453–2462
23 Sprott, G.D. and Jarrell, K.F. (1982) Can. J. Microbiol. 28, 982–986
24 Jarrell, K.F., Colvin, J.R. and Sprott, G.D. (1982) J. Bacteriol. 149, 346–353
25 Shioi, J.-I., Matsuura, S. and Imae, Y. (1980) J. Bacteriol. 144, 891–897
26 Maloney, P.C., Kashket, E.R. and Wilson, T.H. (1975) Methods Membrane Biol. 5, 1–49
27 Harold, F.M. and Altendorf, K.H. (1974) Curr. Top. Membranes Transp. 5, 1–50
28 Harold, F.M., Altendorf, K.H. and Hirata, H. (1974) Ann. N.Y. Acad. Sci. 235, 149–160
29 Heytler, P.G. (1963) Biochemistry 2, 357–361
30 Kashket, E.R., Blanchard, A.G. and Metzger, W.C. (1980) J. Bacteriol. 143, 128–134
31 Sauer, F.D., Erfle, J.D. and Mahadevan, S. (1979) Biochem. J. 178, 165–172
32 Maloney, P.C. (1983) J. Bacteriol. 153, 1461–1470
33 Lolkema, J.S., Hellingwerf, K.J. and Konings, W.N. (1982) Biochim. Biophys. Acta 681, 85–94
34 Thauer, R.K., Jungermann, K. and Decker, K. (1977) Bacteriol. Rev. 41, 100–180
35 Wolfe, R.S. and Higgins, I.J. (1979) Int. Rev. Biochem. 21, 267–300
36 Yamazaki, S. (1982) J. Biol. Chem. 257, 7926–7929
37 McKellar, R.C. and Sprott, G.D. (1979) J. Bacteriol. 139, 231–238
38 Kojima, N., Fox, J.A., Hausinger, R.P., Daniels, L., Orme-Johnson, W.H. and Walsh, C. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 378–382
39 Spencer, R.W., Daniels, L., Fulton, G. and Orme-Johnson, W.H. (1980) Biochemistry 19, 3678–3683
40 Roberton, A.M. and Wolfe, R.S. (1970) J. Bacteriol. 102, 43–51
41 Perski, H.J., Schönheit, P. and Thauer, R.K. (1982) FEBS Lett. 143, 323–326
42 Taylor, G.T. (1982) in Progress in Industrial Microbiology (Bull, M.J., ed.), Vol. 16, pp. 231–329, Elsevier, Amsterdam
43 Sprott, G.D. and Jarrell, K.F. (1981) Can. J. Microbiol. 27, 444–451
44 Zehnder, A.J.B. and Wuhrmann, K. (1977) Arch. Microbiol. 111, 199–205
45 Huser, B.A., Wuhrmann, K. and Zehnder, A.J.B. (1982) Arch. Microbiol. 132, 1–9
46 Elhardt, D. and Böck, A. (1982) Mol. Gen. Genet. 188, 128–134
47 Padan, E., Zilberstein, D. and Rottenberg, H. (1976) Eur. J. Biochem. 63, 533–541
48 Butsch, B. and Bachofen, R. (1982) Experientia 38, 1377