Cation transport mechanisms in *Mycoplasma mycoides* var. Capri cells

Na⁺-dependent K⁺ accumulation

Mohammed BENYUCEF,* Jean-Louis RIGAUD*§ and Gérard LEBLANC†

*Service de Biophysique (Laboratoire associé LA 219 Centre National de la Recherche Scientifique),
Département de Biologie, C.E.N. Saclay, 91191 Gif-Sur-Yvette Cedex, France, and †Laboratoire de Biologie
Marine du C.E.A., 06230 Villefranche-Sur-Mer, France

(Received 5 April 1982/Accepted 17 August 1982)

We have studied some features of K⁺ accumulation by glycolysing *Mycoplasma mycoides* var. Capri cells. We report that when Na⁺ is absent from the external medium, K⁺ accumulates up to the level predicted by the amplitude of the transmembrane electrical potential, $\Delta\psi_m$, measured by Rb⁺ and methyltriphenylphosphonium cation (TPMP⁺) distribution. Therefore, under these experimental conditions, the coupling mechanism of K⁺ uptake consists of a $\Delta\psi_m$ -driven uniport. More important, when Na⁺ is present in the external medium, the level of K⁺ accumulation by glycolysing *Mycoplasma* cells is far too steep to be in equilibrium with $\Delta\psi_m$ (−120 mV for $\Delta\mu_{K^+}$ compared with −90 mV for $\Delta\mu_{Rb^+}$ or $\Delta\mu_{TPMP^+}$). Our results clearly indicate the presence in *Mycoplasma* of an active K⁺-transport system specifically stimulated by Na⁺. Furthermore, by controlling the amplitude of the energy-dependent $\Delta\tilde{\mu}_{H^+}$, we obtain strong evidence that this specific Na⁺-stimulated K⁺ transport is modulated by the transmembrane electrical potential. Finally, we show that ATP is consumed when such a transport system is in activity.

In Mitchell's chemio-osmotic theory (Mitchell, 1968, 1970, 1973), ionic gradients across bacterial cytoplasmic membranes serve the purpose of both the conversion of energy and its transmission from energy-producing to energy-consuming reactions. The gradients generally encountered are those of H⁺, K⁺ and Na⁺ (Harold & Altendorf, 1974, 1977; Lanyi, 1979).

The present and the following paper describe these cation transport systems and their interactions in *Mycoplasma mycoides* var. Capri cells (PG₃ strain).

Previous studies from this laboratory (Benyucef *et al.*, 1981a,b) established that an electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$) was generated across the membranes of these obligatory fermentative micro-organisms during glucose utilization:

Abbreviations used: ΔpH , transmembrane pH gradient; $\Delta\psi_m$, electrical potential; $\Delta\tilde{\mu}_{H^+}$, electrochemical proton gradient; $\Delta\mu_{K^+}$, $\Delta\mu_{Rb^+}$, $\Delta\mu_{TPMP^+}$, chemical potential difference for K⁺, Rb⁺ and TPMP⁺ respectively; TPMP⁺, methyltriphenylphosphonium cation; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide; Mops, 4-morpholinepropanesulphonic acid.

§ To whom correspondence and reprint requests should be sent.

indeed glycolysing *Mycoplasma* cells generate transmembrane ΔpH and $\Delta\psi$ as a result of H⁺ extrusion by the membrane-bound Mg²⁺-dependent ATPase. Most of these observations were explained in terms of the chemio-osmotic concepts. However, it was pointed out at the time (Benyucef *et al.*, 1981a) that the level of the positively-charged K⁺ species accumulation was far higher than the level expected from the amplitude of the electrical potential. Therefore the question arises as to how the active-transport mechanism for K⁺ works to bring about such transmembrane distribution.

The present work is mainly concerned with describing the particular features of the K⁺ transport system in *Mycoplasma* cells. We began by investigating the dependence of the K⁺ transport mechanism on $\Delta\tilde{\mu}_{H^+}$ amplitude. As shown previously (Benyucef *et al.*, 1981b), the regulation of glucose uptake, which leads to a control of the rate of the proton extrusion catalysed by the ATPase, allows a gradation of the respective amplitudes of the $\Delta\tilde{\mu}_{H^+}$ components from the non-energized to the fully-energized states. Under such experimental conditions, it is shown that K⁺ is accumulated up to levels well above those predicted from the magni-

tude of the transmembrane electrical potential, whatever the cell-energization level. Furthermore, the active K^+ transport system, which accumulates K^+ in such a way that $\Delta\mu_{K^+} > \Delta\psi_m$, is shown to be controlled by the transmembrane electrical potential.

We also demonstrate that Na^+ specifically stimulates K^+ transport, thus enabling its distribution to exceed the level predicted by $\Delta\psi$. Lastly, measurements of intracellular ATP levels indicate that some ATP is hydrolysed during the additional K^+ accumulation, i.e. the net K^+ influx mediated by Na^+ requires ATP.

Materials and methods

Organism and growth conditions

Mycoplasma mycoides var. Capri (PG₃ cells) was grown as previously described (Leblanc & Le Grimellec, 1979a; Benyoucef *et al.*, 1981a).

Cells were depleted of internal K^+ by resuspending freshly collected organisms in a K^+ -free buffered-saline solution, either for 30 min at 37°C in the presence of 0.25 μ M-valinomycin or for 2 h at 37°C in the absence of valinomycin. For most experiments we used cells whose K^+ complement had been replaced by Na^+ , by depletion in a K^+ -free buffer containing 100 mM-sodium phosphate, 40 mM-NaCl and 2 mM-MgCl₂, pH 7.2. These cells will hereafter be referred to as ' Na^+ -loaded cells'.

In some experiments we used cells obtained by K^+ depletion in K^+ - and Na^+ -free buffer containing 50 mM-Mops, 17 mM-MgO, 120 mM-choline chloride, 2 mM-MgCl₂ and 2 mM-H₂PO₄, pH 7.2. After incubation, cells were collected by centrifugation and resuspended in the appropriate buffer. These cells will hereafter be referred to as 'choline cells'.

' Na^+ -loaded cells' contained less than 5 mM- K^+ and about 100 mM- Na^+ , as determined by flame photometry. 'Choline cells' contained less than 5 mM- K^+ and 1 mM- Na^+ .

Transport experiments

For measurement of cation uptakes, cells were diluted to 1 mg of protein/ml in the assay buffer and incubated at 37°C for 10 min with 10 mM-glucose. Uptake was initiated by adding $^{42}K^+$ (1 mM), $^{86}Rb^+$ (1 mM) or [^{14}C]TPMP⁺ (27 μ M). Samples (100 μ l) were removed at various intervals, filtered on 0.45 μ m Millipore filters and washed as previously described (Benyoucef *et al.*, 1981a; Leblanc & Le Grimellec, 1979a).

Concentration gradients for the solutes taken up by the cells were calculated on the basis of 4.8 μ l of intracellular water/mg of cell protein (Leblanc & Le Grimellec, 1979a).

Intracellular ATP levels

Cell ATP was extracted by using cold HClO₄

and samples were neutralized with Tris. ATP concentration was assayed (Forest & Walker, 1965) by using firefly-lantern extract in arsenate/Mg²⁺ buffer (Sigma FLE 50).

Protein determinations

Proteins were measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Materials

$^{86}Rb^+$ Cl (86 Ci/mol), $^{42}K^+$ Cl (20 Ci/mol) and [^{14}C]TPMP⁺ iodide (49.2 Ci/mol) were purchased from C.E.N. Saclay, Gif-Sur-Yvette, France.

FCCP (Boehringer), *NN'*-dicyclohexylcarbodiimide (Aldrich-Europe), valinomycin (Sigma) and nigericin (Lilly Lab.) were prepared as concentrated ethanol solutions so that the final ethanol concentration of the cell suspension did not exceed 0.2%.

Results

Relationship between K^+ accumulation, $\Delta\psi_m$ amplitude and ATP levels

In a previous study (Benyoucef *et al.*, 1981b), we showed that in *Mycoplasma* cells, appropriate manipulation of the glucose and α -methylglucose concentrations in the medium can serve to control the respective magnitudes of the electrical potential $\Delta\psi_m$ and the chemical gradient ΔpH , by controlling cell energy production. We used this experimental condition to analyse the relationship between K^+ accumulation and $\Delta\psi_m$ magnitude.

In these experiments, ' Na^+ -loaded cells' in which 95% of the intracellular K^+ had been replaced by Na^+ were incubated in the presence of 0.25 μ M-valinomycin, 10 mM-methyl α -glucoside and glucose at concentrations ranging from 0.1 to 10 mM. The values of the transmembrane electrical potential ($\Delta\psi_m$) were estimated from the equilibrium distribution of [^{14}C]TPMP⁺ or $^{36}Rb^+$ present at 30 μ M and 1 mM respectively (Benyoucef *et al.*, 1981a). Parallel experiments were run with cells incubated in the presence of 1 mM- ^{42}KCl . The steady-state concentration ratios of the three cations across the membrane were converted into potential (mV) by using the Nernst equation and are plotted in Fig. 1(a) as a function of the glucose concentration in the medium.

This Figure shows, first, that $\Delta\mu_{K^+}$, $\Delta\mu_{Rb^+}$ and $\Delta\mu_{TPMP^+}$ were all, without distinction, continuous functions of the external glucose concentration and, more important, that K^+ accumulates well above the levels of Rb^+ and TPMP⁺, regardless of the state of cell energization. The discrepancy is maximal in the fully energized state, i.e. at glucose concentrations about 1 mM, where $\Delta\mu_{K^+}$ is -120 mV, compared

Fig. 1. K^+ , Rb^+ and TPMP^+ distribution in Na^+ -loaded *Mycoplasma* cells

Fresh cells were previously depleted of their internal K^+ in a K^+ -free buffer containing 100 mM-sodium phosphate, 40 mM- NaCl and 2 mM- MgCl_2 , pH 7.2, in the presence of 0.25 μM -valinomycin, as described in the Materials and methods section. These Na^+ -loaded cells were then incubated for 10 min at 37°C in the same buffer, in the presence of 10 mM-methyl α -glucoside, 0.25 μM -valinomycin and $^{42}\text{K}^+$ (1 mM; sp. radioactivity 9 Ci/mol), $^{86}\text{Rb}^+$ (1 mM; sp. radioactivity 4.1 Ci/mol) or ^{14}C - TPMP^+ (30 μM ; sp. radioactivity 50 Ci/mol). Finally, cation uptake was initiated by adding glucose at given concentrations. Cation distribution at the steady state was determined by filtration assay as described in the Materials and methods section and converted into mV by using the Nernst equation. (a) shows K^+ (Δ).

with -90 mV for $\Delta\mu_{\text{Rb}^+}$ or $\Delta\mu_{\text{TPMP}^+}$. This difference is highly significant, since in the case of K^+ the internal concentration at equilibrium was 100 mM, compared with 33 mM for Rb^+ , K^+ and Rb^+ being present in the medium at a concentration of 1 mM.

The relationship between $\Delta\mu_{\text{K}^+}$ and $\Delta\psi_m$, taken as being the same as $\Delta\mu_{\text{Rb}^+}$ (Benyoucef *et al.*, 1981a), is shown in Fig. 1(b). From the linear correlation observed, it can be concluded that, although K^+ accumulation is not in equilibrium with $\Delta\psi_m$, it is linearly dependent on the magnitude of the electrical potential. A similar correlation can be established between $\Delta\mu_{\text{TPMP}^+}$ and $\Delta\mu_{\text{K}^+}$. However, the somewhat large correction of $\Delta\mu_{\text{TPMP}^+}$ for non-specific contribution, due to the binding of the probe to the membrane (Ghazi *et al.*, 1981; Michel & Oesterhelt, 1980), makes this second correlation less conclusive. Further, such a comparison between $\Delta\mu_{\text{K}^+}$ and $\Delta\psi_m$ (or $\Delta\mu_{\text{Rb}^+}$ or $\Delta\mu_{\text{TPMP}^+}$) is valid only if the experiments are performed under strictly identical experimental conditions. However, this ideal condition cannot be satisfied for the following technical reasons (Benyoucef *et al.*, 1981a): $\Delta\mu_{\text{K}^+}$ is measured with KCl in the medium but without RbCl , and $\Delta\psi_m$ is measured with RbCl but without KCl in the medium. These considerations might have important consequences, since, on the one hand, (a) the transmembrane electrical potential $\Delta\psi_m$ is generated as a consequence of H^+ extrusion by an ATP-requiring system, i.e. the membrane-bound Mg^{2+} -dependent ATPase (Benyoucef *et al.*, 1981a,b; Leblanc & Le Grimellec, 1979b) and (b) intracellular ATP levels are exclusively controlled by substrate phosphorylation, i.e. glycolysing activity, since *Mycoplasma mycoides* var. Capri cells are obligatory fermentative anaerobic micro-organisms (Razin, 1973). On the other hand, data reported in the literature (Lardy & Ziegler, 1945; Neijssell & Tempest, 1979; Harold & Baarda, 1979; Kroll & Booth, 1981) indicate that cytoplasmic K^+ stimulates the glycolytic activity and consequently regulates the cell ATP level in a variety of micro-organisms. Thus, under our experimental conditions, ATP levels might have differed, depending on the presence or absence of KCl . We therefore

Rb^+ ($\circ$) and TPMP^+ ($\bullet$) distribution as a function of cell energization. All experiments were performed on the same preparation of Na^+ -loaded cells. We have corrected the values of TPMP^+ accumulation for non-specific fixation of this cation to the membranes. This binding was calculated from the difference between TPMP^+ and Rb^+ or K^+ accumulation in non-energized cells. (b) shows the relationships between K^+ and Rb^+ distribution. Cation distributions were taken from Fig. 1(a). The broken line represents the identity line expected for similar cation distribution.

Fig. 2. Effect of K^+ or Rb^+ on ATP pools in glycolysing Na^+ -loaded cells

Na^+ -loaded cells were resuspended (1 mg/ml) in a buffer containing 100 mM-sodium phosphate, 40 mM- $NaCl$ and 2 mM- $MgCl_2$, pH 7.2. After 10 min incubation at $37^\circ C$ with 1 mM- KCl (□) or without KCl (●), 10 mM-glucose was added and cell ATP contents were determined at various times. At the times indicated by the arrows, a pulse of 1 mM- KCl (Δ) or 1 mM- $RbCl$ (○) was administered to the Na^+ -loaded cells resuspended in phosphate buffer devoid of Rb^+ and K^+ .

determined the intracellular ATP levels in the experimental conditions under which $\Delta\mu_{K^+}$ and $\Delta\mu_{Rb^+}$ (i.e. $\Delta\psi_m$) were measured.

Fig. 2 shows the time course of cell ATP formation after addition of 10 mM-glucose to Na^+ -loaded cells resuspended in different buffers. In sodium phosphate buffer containing 1 mM- KCl , the intracellular ATP level reached an equilibrium value of 8 mM instead of 2–3 mM in the absence of additional KCl . When KCl was replaced by $RbCl$, ATP levels reached also a value of 2.5 mM (results not shown), which was not significantly different from the level found when either KCl or $RbCl$ was omitted. Addition of 1 mM- KCl to Na^+ -loaded cells quickly raised the intracellular level to 8 mM, whereas addition of 1 mM- $RbCl$ to the cells resuspended in the absence of K^+ did not change the ATP levels. All these results effectively show that the presence of KCl but not of $RbCl$ in the cell suspension, and very probably in the cytoplasm, increases intracellular ATP levels considerably. The immediate question arising is whether, when Rb^+ accumulation is measured in the absence of KCl , the electrical potential is fully generated across the *Mycoplasma* membrane, i.e. do ATP levels limit the amplitude of $\Delta\psi_m$ in $RbCl$ -incubated cells? Examination of Figs. 3 and 4 shows that this is not the case. These Figures indicate the cellular ATP

Fig. 3. Effects of KCl and $RbCl$ on intracellular ATP level as a function of cell energization

Na^+ -loaded cells (1 mg/ml) were incubated at $37^\circ C$ for 10 min in the presence of 10 mM-methyl- α -glucoside in a phosphate buffer containing 1 mM- KCl (◆) or 1 mM- $RbCl$ (●). Cell ATP contents were determined after 30 min incubation in the presence of various glucose concentrations.

levels measured in the presence of methyl α -glucoside and various glucose concentrations in Na^+ -loaded cell incubated in the presence of either 1 mM- KCl or 1 mM- $RbCl$.

It is clear from Fig. 3 that only when glucose concentrations exceed 1 mM are intracellular ATP concentrations much higher in the presence of KCl than in that of $RbCl$. Below this glucose concentration, cellular ATP levels are similar in $RbCl$ - and KCl -incubated cells, although K^+ accumulation is far greater than that of Rb^+ . Furthermore, the relationships between the respective amplitudes of cation accumulation and the ATP levels can be deduced from the Rb^+ and K^+ distributions given in Fig. 1(a) and ATP levels indicated in Fig. 3. Fig. 4 shows, first, that whatever the cell ATP concentration, K^+ accumulation always exceeds that of Rb^+ and, secondly, that 2 mM-ATP is sufficient to generate maximal accumulation of each cation; increasing ATP levels above this concentration of 2 mM do no increase cation accumulation. This leads to the conclusion that: (i) greater accumulation of K^+ than of Rb^+ cannot be related to the limitation of intracellular ATP levels; (ii) $\Delta\psi_m$, measured by Rb^+ distribution at high glucose concentrations, corresponds to the maximal transmembrane electrical-potential value that can be generated across the membrane independently of the presence of external K^+ . Thus, KCl enhances intracellular ATP levels but not $\Delta\psi_m$ amplitudes [this should be linked up with the fact that the amplitude of fluorescence quenching of Merocyanine 540 was found to be independent of the presence of 1 mM- KCl (Benyoucef *et al.*, 1981b)].

Fig. 4. Relationship between K^+ or Rb^+ distribution and ATP levels

Rb^+ (○) and K^+ distributions (△) were taken from Fig. 1(a). ATP levels measured under the same experimental conditions were taken from Fig. 3.

Na^+ -dependent K^+ uptake

The general pattern of K^+ movement in PG₃ cells was established by previous studies (Leblanc & Le Grimellec, 1979a,b) in which we found that when cells were resuspended in a phosphate-buffered solution containing 1 mM-KCl, they lost their internal K^+ by substitution with Na^+ and H^+ . After glucose addition, the cells accumulated K^+ and extruded Na^+ and H^+ . As an approach to this problem, we attempted to find a possible link between Na^+ and K^+ movements.

In order to study the influence of Na^+ on K^+ reaccumulation, we used 'choline cells', i.e. cells depleted of their internal K^+ by incubation in a choline buffer containing no K^+ or Na^+ (see the Materials and methods section). Fig. 5 shows the time course and extent of K^+ accumulation by these cells resuspended in buffered solution with or without Na^+ . In the presence of Na^+ in the external medium, upon addition of 10 mM-glucose, the intracellular K^+ concentration reached an equilibrium value of about 100 mM. In the absence of Na^+ from the external medium, the intracellular K^+ concentration reached an equilibrium value of only 30 mM. Importantly, addition of 10 mM-NaCl at the plateau induced K^+ reaccumulation to a level comparable with that measured in cells immediately

Fig. 5. Effect of Na^+ on K^+ accumulation by 'choline cells' (a) and Na^+ -concentration-dependence of K^+ accumulation by 'choline cells' (b)

(a) Fresh cells were previously depleted of their internal K^+ in a choline-buffered saline solution, as described in the Materials and methods section. These 'choline cells' were then resuspended at 1 mg/ml in either a buffer containing 50 mM-Mops, 17 mM-MgO, 120 mM-NaCl, 2 mM-MgCl₂ and 2 mM-H₃PO₄, pH 7.2, (●) or an Na^+ -free buffer (▲) in which NaCl was replaced by 120 mM-choline chloride. After 10 min incubation at 37°C in the presence of 10 mM-glucose, cation uptake was initiated by adding $^{42}K^+$ (1 mM, sp. radioactivity 9 Ci/ml) and labelled cation accumulation was measured as a function of time by filtration assay and converted into mM, taking 4.8 μ l of cell water/mg of cell protein as the intracellular cell volume. After K^+ distribution had reached equilibrium, a pulse of 10 mM-NaCl was administered at the time indicated by the arrow (----). (b) Experimental conditions were as in (a). 'Choline cells' were resuspended in a choline-buffered solution (i.e. Na^+ -free) in the presence of 10 mM-glucose. At time zero, cation uptake was initiated by adding $^{42}K^+$ (1 mM, sp. radioactivity 9 Ci/mol). At the time indicated by the arrow, the suspension was divided into four portions to which were added 0.1 mM- (□), 0.5 mM- (■), 1 mM- (△) and 10 mM-NaCl (▲) respectively.

Table 1. *Effects of the suspension medium on the levels of K^+ and Rb^+ accumulation by 'choline cells'*
 'Choline cells' were prepared in the absence of valinomycin as described in the Materials and methods section, resuspended at 1 mg of cell protein/ml in the buffers (pH 7.2) listed in the Table, and incubated at 37°C with 10 mM-glucose in the presence or absence of 0.25 μ M-valinomycin. Cation uptake was initiated by adding $^{86}Rb^+$ (1 mM) or $^{32}K^+$ (1 mM). Cation accumulation at the steady state was determined by filtration. Mops-choline buffer: 50 mM Mops, 17 mM-MgO, 120 mM-choline chloride, 2 mM-MgCl₂ and 2 mM-H₃PO₄. Mops-Na⁺ and Mops-Li⁺ buffers: as for Mops-choline buffer, but 120 mM-NaCl or 120 mM-LiCl replaced choline chloride. Sodium phosphate buffer: 100 mM-sodium phosphate, 40 mM-NaCl and 2 mM-MgCl₂.

Suspension medium	$^{86}Rb^+$ accumulation in the presence of 0.25 μ M-valinomycin (mM)	$^{42}K^+$ accumulation (mM)	
		+0.25 μ M-Valinomycin	No valinomycin
Mops-choline	38	35	30
Mops-choline + 10 mM-NaCl	38	96	100
Mops-choline + 10 mM-LiCl	35	34	—
Mops-Na ⁺	30	125	110
Mops-Li ⁺	35	34	—
Sodium phosphate	30	100	100

Table 2. *Effects of the suspension medium on the levels of K^+ and Rb^+ accumulation by Na⁺-loaded cells*
 Experimental conditions were as described in the legend to Table 1, except that 'choline cells' were replaced by Na⁺-loaded cells.

Suspension medium	$^{86}Rb^+$ accumulation in the presence of 0.25 μ M-valinomycin (mM)	$^{42}K^+$ accumulation (mM)	
		+0.25 μ M-Valinomycin	No valinomycin
Mops-choline	31	35	30
Mops-choline + 10 mM-NaCl	31	80	85
Mops-Na ⁺	35	100	96
Sodium phosphate	30	96	105

incubated in the presence of Na⁺. The inset in Fig. 5 clearly indicates that stimulation of K⁺ transport by Na⁺ is concentration-dependent between 0.1 and 10 mM-NaCl. The maximal stimulation was observed at 10 mM-NaCl.

When Rb⁺ accumulated under the same conditions, no difference in the intracellular Rb⁺ concentration was observed in the presence or absence of Na⁺ in the buffer (Table 1), or after administration of a pulse of 10 mM-NaCl (results not shown).

Tables 1 and 2 give all the data obtained on both choline cells and Na⁺-loaded cells. Under all the conditions tested, Rb⁺ reached an equilibrium value of 30 mM, which corresponds to a transmembrane electrical potential of about -90 mV. In the absence of Na⁺ from the external medium, K⁺ accumulated up to the same level as Rb⁺, and therefore to the level expected from the transmembrane electrical potential. The presence of Na⁺ in the external buffer raised K⁺ accumulation to a level well above that of Rb⁺ (approx. 100 mM compared with 30 mM) without changing $\Delta\psi_m$.

Contrary to Rb⁺ accumulation, which only occurred in glycolysing *Mycoplasma* in the presence of 0.25 μ M-valinomycin (Benyoucef *et al.*, 1981a),

K⁺ accumulation occurred regardless of the presence or absence of this antibiotic. Furthermore, K⁺ accumulation levels were similar both in the presence and absence of 0.25 μ M-valinomycin.

Results in Table 1 indicate that resuspension of 'choline cells' in a buffer containing up to 140 mM-LiCl did not stimulate K⁺ uptake, which amounted to about 30 mM. It can therefore be concluded that, in our system, Li⁺ cannot replace Na⁺ and that we are dealing specifically with an Na⁺-stimulated K⁺ transport system.

Another point to be stressed is that this Na⁺ stimulation was observed to the same extent, regardless of whether the cells were depleted of their internal K⁺ in the presence or absence of Na⁺ (Na⁺-loaded cells or 'choline cells'; Tables 1 and 2). When the Na⁺-loaded cells were resuspended in a choline chloride buffer, K⁺ uptake was 30–40 mM; addition of 10 mM-NaCl at the plateau raised the K⁺ uptake to 100 mM, as observed for 'choline cells' (Fig. 5). This suggests either that Na⁺ enters the cells, thus allowing stimulation of K⁺ accumulation, or that Na⁺ has some external effect. The results given in Fig. 6 corroborate the first hypothesis. This Figure indicates that the Na⁺-stimulation of K⁺ transport requires P_i. When Na⁺-loaded cells were

Fig. 6. Effects of phosphate on Na^+ -dependent K^+ accumulation

Na^+ -loaded cells were resuspended at 1 mg/ml in a Mops-choline-buffered saline solution (pH 7.2) in the presence ($\blacktriangle$) or absence ($\bullet$) of 2 mM- H_3PO_4 . After 10 min incubation at 37°C with 10 mM-glucose, cation uptake was initiated by adding $^{42}\text{K}^+$ (1 mM, sp. radioactivity 9 Ci/mol). When steady-state K^+ accumulation was reached, 10 mM- NaCl was added to the cell suspensions at the time indicated by the arrow and the changes in K^+ accumulation were monitored. The inset shows the effects of Na^+ on phosphate uptake. Experimental conditions were as above, except that the Mops-choline-buffered solution was supplemented with 1 mM-KCl. After 10 min incubation with 10 mM-glucose, $^{32}\text{P}_i$ (carrier free, 2 mM) was added to the cell suspension and its accumulation was measured by filtration assay. At time indicated by the arrow, a pulse of 10 mM- NaCl was administered.

resuspended in buffers devoid of Na^+ but in the presence or absence of P_i , they only accumulated 30–40 mM- K^+ after glucose addition. However, it is true that addition of 10 mM- NaCl at the plateau caused a rapid increase in K^+ level up to 100 mM only when phosphate is present in the external medium. Control experiments indicated (results not shown) that (a) a further addition of 2 mM- P_i after the NaCl pulse to cells incubated in the absence of phosphate induced an increase in K^+ accumulation up to 100 mM; (b) addition of 2 mM- P_i in the absence of NaCl in the medium did not increase K^+ accumulation, suggesting that P_i is not directly involved in the stimulation of K^+ accumulation. Furthermore, in an Na^+ -free medium the limitation of K^+ accumulation in the absence or presence of P_i was not due to limited ATP availability; indeed in the absence of P_i cell ATP amounted to 2.5 mM and in its presence to 5.5 mM (Fig. 7). Although different, those ATP levels were high enough to permit the

Fig. 7. Effects of K^+ , N^+ and P_i on intracellular ATP levels

'Choline cells' were resuspended in buffers containing basically 50 mM-Mops, 17 mM-MgO, 120 mM-choline chloride and 2 mM- MgCl_2 (pH 7.2), and supplemented with 1 mM-KCl, 10 mM- NaCl , 2 mM- H_3PO_4 , or a combination of these compounds after additional 10 mM-glucose. Intracellular ATP levels were determined at the steady state. The inset shows the time course of ATP consumption after administration of a pulse of 10 mM- NaCl . 'Choline cells' were resuspended in a choline-Mops buffer containing 1 mM-KCl and 2 mM- H_3PO_4 , pH 7.2. Intracellular levels were determined as a function of time after addition of 10 mM-glucose ($\circ$). At the time indicated by the arrow, a pulse of 10 mM- NaCl was applied ($\bullet$).

largest K^+ accumulation observed of 100–120 mM (see Fig. 4). Thus, the results presented in Fig. 6 point to the conclusion that, to be effective, Na^+ stimulation of K^+ transport requires P_i in the external medium. The inset in Fig. 6 indicates that, conversely, the phosphate uptake induced by glucose addition was also stimulated by the presence of Na^+ in the external bathing solution.

Taken together, these experiments suggest the existence of a clear link between Na^+ and P_i , which in turn seems to indicate that to stimulate K^+ transport effectively, Na^+ must first enter the cells as sodium phosphate.

Finally, comparison of intracellular ATP levels in glycolysing cells incubated in media with or without K^+ and/or Na^+ suggests that the Na^+ -dependent K^+ accumulation is an ATP-consuming process (Fig. 7). Thus, when NaCl and KCl were omitted from a phosphate-containing medium, cell ATP amounted to 4.5 mM. Since comparable ATP concentrations were found in cells incubated with 10 mM-NaCl, it can be concluded that Na^+ did not in itself affect the ATP level. A completely different effect was observed when NaCl was added to cells incubated with 1 mM-KCl: whereas the ATP level of K^+ -incubated cells reached 5.5 mM, the cells resuspended in the presence of both KCl and NaCl had a significantly lower intracellular ATP concentration (3.5 mM). The combined results of all these experiments support the contention that ATP is consumed when the Na^+ -dependent K^+ accumulation process is active. The inset in Fig. 7 shows the time course of ATP consumption when NaCl was added to glycolysing cells in a buffer already containing 1 mM-KCl. After addition of 10 mM-NaCl, the ATP level dropped from 5.5 mM to 3.5 mM, with a $t_{1/2}$ of about 4 min.

Discussion

The experiments described in the present paper define a number of important features of K^+ transport in *Mycoplasma mycoides* var. Capri cells: (i) K^+ ions are actively accumulated by a transport system controlled by the transmembrane electrical potential; (ii) the entry of K^+ in excess of the amount predicted by $\Delta\psi_m$ is absolutely dependent on the presence of Na^+ ; and (iii) this Na^+ -dependent K^+ uptake is an ATP-consuming system.

The present data confirm (Benyoucef *et al.*, 1981a,b) that K^+ accumulation in *Mycoplasma* cells leads to values of $\Delta\mu_{K^+}$ more negative than those that can be accounted for by passive uniport in response to a transmembrane electrical potential ($\Delta\psi_m$) measured with Rb $^+$ or TPMP $^+$. In the fully energized state, i.e. in the presence of 10 mM-glucose, $\Delta\mu_{K^+}$ amounts to approx. -120 mV, compared with a $\Delta\psi_m$ of -90 mV. This represents a 3-fold difference in the cation concentration (100 mM- K^+ compared with 30 mM-Rb $^+$).

The large difference found in the ATP content of cells incubated with 1 mM-KCl or -RbCl (Fig. 2) may cast doubt on the validity of extrapolating $\Delta\psi_m$ values from one experimental condition to another since the low level of cell ATP production in RbCl-incubated cells (2.5 mM) compared with that found in KCl-incubated cells (8 mM) might have limited the amplitude of $\Delta\psi_m$. However, such a possibility is very unlikely.

In this connection Fig. 4 indicates that intra-

cellular ATP levels of 2 mM are sufficient to develop a maximal $\Delta\psi_m$ across *Mycoplasma* membranes, and that a further increase in ATP levels does not involve any increase in $\Delta\psi_m$. Thus, although the presence of KCl in the suspension medium markedly enhances intracellular ATP levels (Fig. 2), it does not lead to a change in the amplitude of the electrical potential. It should be emphasized that $\Delta\mu_{K^+}$ was significantly higher than $\Delta\psi_m$ at all the intracellular ATP levels measured. Importantly, although $\Delta\mu_{K^+} > \Delta\psi_m$, the K^+ accumulation process is controlled by this transmembrane potential. The control of energy transduction mechanism achieved by controlling the glycolytic activity of *Mycoplasma* cells (Benyoucef *et al.*, 1981b) allows formulation of this important conclusion about the relationship between $\Delta\mu_{K^+}$ and cell energy production. Fig. 1(a) indicates that K^+ movements across the membrane depend on cell energization. The amplitude of $\Delta\mu_{K^+}$ progressively varies from the non-energized to the fully energized state. Even more important is the information provided by Fig. 1(b), i.e. that K^+ distribution is linearly related to the amplitude of the transmembrane electrical potential. At this point, it is important to recall that, besides $\Delta\psi_m$, the electrical component of $\Delta\tilde{\mu}_{H^+}$, a transmembrane pH gradient is maintained across the membrane of glycolysing *Mycoplasma* cells (Benyoucef *et al.*, 1981a). This immediately raises the question of the relative contribution of ΔpH to K^+ transport. Two lines of evidence suggest that ΔpH is not essential for this transport. (i) In the presence of 10 mM-methyl α -glucoside, 1 mM-glucose was sufficient to promote maximal K^+ accumulation and maximal $\Delta\psi_m$ (Fig. 1a), although only a small ΔpH was generated across *Mycoplasma* membranes at this concentration [at 1 mM-glucose, 80–90% of the maximal $\Delta\psi_m$ was generated, whereas no ΔpH is measurable (Benyoucef *et al.*, 1981b)]. (ii) In our *Mycoplasma* strain, FCCP did not change Rb $^+$ and TPMP $^+$ distribution levels as well as the amplitude of Merocyanine 540 fluorescence quenching (Leblanc & Le Grimellec, 1979b; Benyoucef *et al.*, 1981a; results not shown), although it did abolish the ΔpH (Benyoucef *et al.*, 1981a,b; Le Grimellec *et al.*, 1982; the following paper). [The inefficiency of FCCP to collapse $\Delta\psi$ at pH 7.3 can be explained by taking into account the low electrical capacitance of the membrane, the pK of the protonophore and/or the negative charge of the phospholipids. In this context, when *Mycoplasma* are resuspended in a buffer at pH 5.6, FCCP collapses the electrical potential, which amounted to only -60 mV before addition of the uncoupler (Benyoucef *et al.*, 1981a; results not shown).] Since K^+ distribution is not changed in the presence of FCCP, we thus conclude that the K^+ transport system is activated by the transmembrane potential.

The second point to emphasize from this study is that, to be effective, the active $\Delta\psi$ -dependent K^+ transport system requires Na^+ (Fig. 5; Tables 1 and 2).

Our results allow definition of several important features of the K^+ uptake dependence on Na^+ . In the absence of Na^+ from the suspension medium, K^+ -depleted cells accumulated the same levels of K^+ and Rb^+ . This observation might support the hypothesis that a uniport mechanism with internally negative membrane potential drives K^+ transport (Mitchell, 1968). However, addition of 10 mM-NaCl to the external medium leads to a 3-fold increase in K^+ accumulation. The inset in Fig. 5 indicates that this Na^+ stimulation of K^+ transport is concentration-dependent, the approximate K_m being estimated at about 1 mM and the maximal effects observed at 10 mM. Our results show that K^+ uptake is stimulated by Na^+ but not by Li^+ (Table 1). On the other hand, to be effective, Na^+ requires the presence of phosphate in the medium (Fig. 6). If we consider that, conversely, the phosphate entry is dependent on the presence of Na^+ (inset Fig. 6), it can be assumed that the Na^+ -stimulation of K^+ transport is secondary to uptake of sodium phosphate.

The Na^+ stimulation of K^+ uptake was observed in the absence of valinomycin (i.e. under more physiological conditions). Table 1 indicates that in the absence of NaCl, K^+ accumulation was similar, whether or not the cells were pretreated with valinomycin. Further, $\Delta\mu_{K^+}$ amplitude was similar to that of $\Delta\mu_{Rb^+}$, i.e. to the $\Delta\psi_m$ amplitude measured in the presence of valinomycin. Since qualitative estimates of the electrical potential using Mero-cyanine 540 showed that the glucose-induced change in fluorescence intensity was not affected by valinomycin (Benyoucef *et al.*, 1981a,b; Leblanc & Le Grimellec, 1979b) it can be concluded that when Na^+ is absent from the medium $\Delta\mu_{K^+}$ is equal to $\Delta\psi_m$, even in the absence of valinomycin. This suggests the presence of a K^+ uniport system in the membranes. The relationships between this apparent mechanism of uniport in the absence of Na^+ , on the one hand, and the active Na^+ -dependent K^+ uptake, on the other, requires further investigation.

The last point worth stressing in the present findings is the drop in intracellular ATP level occurring concomitant with the stimulation of K^+ uptake by Na^+ . As shown in Fig. 7, Na^+ does not modify the intracellular levels, whereas K^+ noticeably raises them. However, when Na^+ and K^+ are present in the medium, the ATP level drops. The inset in Fig. 7 shows the time course of this ATP consumption on a pulse of Na^+ , i.e. in conditions under which the Na^+ -dependent K^+ uptake occurs (Fig. 5).

In conclusion, the present study shows that K^+ is

transported into *Mycoplasma mycoides* var. Capri cells by an active mechanism, modulated by the transmembrane electrical potential; this mechanism is specifically dependent on the presence of Na^+ , and is ATP-consuming. Taken together, these properties suggest that the K^+ transport system of *Mycoplasma* cells differs from the systems described for other micro-organisms such as *Streptococcus faecalis* (Bakker & Harold, 1980), *Streptococcus lactis* (Kashket, 1979) and *Escherichia coli* (Epstein *et al.*, 1978; Sorensen & Rosen, 1980; Epstein & Laimins, 1980; Bakker, 1982).

Although the results presented in this paper clearly show that Na^+ stimulates the K^+ transport system in *Mycoplasma* cells, the mechanism by which this stimulation occurs remains to be defined. In principle, cation dependence may be caused by: (a) influence of the cation on the driving force for transport, i.e. $\Delta\psi_m$; (b) participation of Na^+ in the translocation across the membrane (symport or antiport). Our results indicate that Na^+ stimulation of K^+ transport is not related to any modification of the $\Delta\psi_m$ amplitude (Tables 1 and 2 show that this $\Delta\psi_m$ amplitude is not affected by the addition of NaCl). In the following paper, we therefore focus our attention on the link between the movements of Na^+ and K^+ and also H^+ .

References

- Bakker, E. P. (1982) *Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria* (Palmieri, P., Quagliariello, E., Siliprandi, H. & Slater, E. C., eds.), Elsevier/North-Holland, Amsterdam.
- Bakker, E. P. & Harold, F. (1980) *J. Biol. Chem.* **255**, 433–440.
- Benyoucef, M., Rigaud, J. L. & Leblanc, G. (1981a) *Eur. J. Biochem.* **113**, 491–498.
- Benyoucef, M., Rigaud, J. L. & Leblanc, G. (1981b) *Eur. J. Biochem.* **113**, 499–506.
- Epstein, W. & Laimins, L. (1980) *Trends Biochem. Sci.* **5**, 21–23.
- Epstein, W., Whitelaw, V. & Hesse, J. (1978) *J. Biol. Chem.* **253**, 6666–6668.
- Forest, W. W. & Walker, D. J. (1965) *J. Bacteriol.* **89**, 1448–1452.
- Ghazi, A., Schechter, E., Letellier, L. & Labedau, B. (1981) *FEBS Lett.* **125**, 197–200.
- Harold, F. M. & Altendorf, K. (1974) *Curr. Top. Membr. Transp.* **5**, 1–50.
- Harold, F. M. & Altendorf, K. (1977) *Bioenergetics* **6**, 83–149.
- Harold, F. M. & Baarda, J. R. (1979) *J. Biol. Chem.* **254**, 2261–2268.
- Heefner, D. L., Kobayashi, H. & Harold, F. M. (1980) *J. Biol. Chem.* **255**, 11403–11407.
- Kashket, E. R. (1979) *J. Biol. Chem.* **254**, 8129–8131.
- Kroll, R. G. & Booth, I. R. (1981) *Biochem. J.* **198**, 691–698.

- Lanyi, J. K. (1979) *Biochim. Biophys. Acta* **559**, 377–397
- Lardy, H. & Ziegler, J. (1945) *J. Biol. Chem.* **159**, 343–345
- Leblanc, G. & Le Grimellec, C. (1979a) *Biochim. Biophys. Acta* **554**, 156–167
- Leblanc, G. & Le Grimellec, C. (1979b) *Biochim. Biophys. Acta* **554**, 168–179
- Le Grimellec, C., Lajeunesse, D. & Rigaud, J. L. (1982) *Biochim. Biophys. Acta* **687**, 281–290
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Michel, H. & Oesterhelt, D. (1980) *Biochemistry* **19**, 4607–4614
- Mitchell, P. (1968) *Chemiosmotic Coupling and Energy Transduction*, Glynn Research, Bodmin, Cornwall
- Mitchell, P. (1970) in *Membranes and Ion Transport* (Bittar, E. E., ed.), vol. 1, pp. 192–256, Wiley Interscience, New York
- Mitchell, P. (1973) *Bioenergetics* **4**, 63–91
- Neijssell, O. M. & Tempest, D. W. (1979) *Microbial Technology* (Bull, A. T., Ellwood, D. C. & Ratledge, C., eds.), pp. 53–82, Cambridge University Press, Cambridge
- Razin, S. (1973) *Adv. Microb. Physiol.* **10**, 1–80
- Sorensen, E. M. & Rosen, B. P. (1980) *Biochemistry* **19**, 1458–1462