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Role of Na^+ Cycle in Cell Volume Regulation of *Mycoplasma gallisepticum*

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The mechanism for the extrusion of Na^+ from *Mycoplasma gallisepticum* cells was examined. Na^+ efflux from cells was studied by diluting $^{22}Na^+$-loaded cells into an isoosmotic NaCl solution and measuring the residual $^{22}Na^+$ in the cells. Uphill $^{22}Na^+$ efflux was found to be glucose dependent and linear with time over a 60-s period and showed almost the same rate in the pH range of 6.5 to 8.0. $^{22}Na^+$ efflux was markedly inhibited by dicyclohexylcarbodiimide (DCCD, 10 μM), but not by the proton-conducting ionophores SF6847 (0.5 μM) or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 10 μM) over the entire pH range tested. An ammonium diffusion potential and a pH gradient were created by diluting intact cells or sealed membrane vesicles of *M. gallisepticum* loaded with NH_4Cl into a choline chloride solution. The imposed H^+ gradient (inside acid) was not affected by the addition of either NaCl or KCl to the medium. Dissipation of the proton motive force by CCCP had no effect on the growth of *M. gallisepticum* in the pH range of 7.2 to 7.8 in an Na^+-rich medium. Additionally, energized *M. gallisepticum* cells were stable in an isoosmotic NaCl solution, even in the presence of proton conductors, whereas nonenergized cells tended to swell and lyse. These results show that in *M. gallisepticum* Na^+ movement was neither driven nor inhibited by the collapse of the electrochemical gradient of H^+, suggesting that in this organism Na^+ is extruded by an electrogenic primary Na^+ pump rather than by an Na^+-H^+ exchange system energized by the proton motive force.

Mycoplasmas are among the simplest of autonomously replicating organisms (16). Without a rigid peptidoglycan-based cell wall or actin-based cytoskeleton structure, these organisms rely for the regulation of their cell volume on mechanisms to extrude solutes that enter the cell (7, 8, 12, 20, 25). Without such mechanisms, the cells swell and eventually lyse. It has been observed in *Acholeplasma laidlawii* (6) and *Mycoplasma gallisepticum* (20) that when cells are incubated in an isoosmotic solution of NaCl in the absence of an energy source, the cells slowly lyse; however, lysis was prevented by replacing NaCl with impermeant compounds such as sucrose, or by the addition of an energy source (6, 20). It was suggested (6, 21) that swelling was the result of NaCl entering the cell and that volume regulation depends on NaCl extrusion.

Bacteria have been proposed to have an Na^+/H^+ antiporter to extrude Na^+ (26). Na^+/H^+ antiporter activity drives sodium across a membrane by coupling its movement to that of protons moving down their chemical potential (proton motive force, $\Delta\tilde{\mu}H^+$). This exchange must therefore be coupled to a primary mechanism that is generating $\Delta\tilde{\mu}H^+$. As mycoplasmas do not have cytochromes (18), they rely on glucose for ATP production, which in turn is used in the generation of $\Delta\tilde{\mu}H^+$. Mitchell (13, 14) first suggested that the mechanism by which bacteria maintain low intracellular levels of Na^+ is a tightly coupled Na^+-H^+ exchange mechanism. Tests for such a mechanism have traditionally used proton-conducting ionophores, which act to deplete or run down $\Delta\tilde{\mu}H^+$ and therefore inhibit exchange. Na^+ extrusion was consistently shown to be inhibited by uncouplers in various bacteria (5). Also, in *M. gallisepticum*, it was suggested that the extrusion of Na^+ occurs through the combined operation of a dicyclohexylcarbodiimide-sensitive H^+-ATPase and an Na^+/H^+ antiporter (8–10). Nevertheless, no direct evidence was presented that Na^+ movements across the membrane were driven by $\Delta\tilde{\mu}H^+$, the proposed driving force. Experiments were therefore designed to test whether cellular swelling was due to the entrance of Na^+ into the cell and whether the mechanism for Na^+ extrusion was a secondary Na^+/H^+ antiporter. The data obtained with cells and membrane vesicles are not consistent with the proposed antiporter model (8–10). It is proposed that *M. gallisepticum* regulates its intracellular volume by active Na^+ extrusion through a primary transport mechanism.

MATERIALS AND METHODS

Cell and membrane preparations. *Mycoplasma gallisepticum* A5969 cells were grown in a modified Edward medium (19) supplemented with 4% horse serum and 0.5% glucose and adjusted to pH 8.0. For radioactive labeling of membrane lipids, cells were grown with 0.004 μCi of [^{3}H]palmitate per ml. The growth medium was inoculated with a 0.1 to 2% frozen inoculum and incubated vertically at 37°C for 18 to 26 h. Growth was monitored by measuring the A_{640} and pH changes of the growth medium. Cells were harvested by centrifugation at 12,000 × *g* for 10 min, washed, and suspended in isoosmotic solutions of either sodium chloride (0.25 M), sucrose (0.5 M), glycylglycine chloride (0.25 M), or choline chloride (0.25 M). For swelling measurements, cells were harvested at the late exponential or stationary phase of growth (A_{640}, 0.27 to 0.30). The viability of the cell suspensions was measured by the colony-counting technique (1). Protein in the cell suspension was measured by the method of Lowry et al. (11), with bovine serum albumin as a standard.

Membranes were isolated following the lysis of glycerol-loaded cells as described previously (19). Sealed membrane vesicles were prepared by fusing *M. gallisepticum* membranes with phosphatidylcholine (type IIs; Sigma Chemical Co.)-cholesterol (25 mol%) lipid vesicles. Fusion was in-

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duced by the quick freeze–slow thaw sonication procedure (2).

Measurement of intracellular water volume. To determine intracellular volume, cells were incubated in 2.4 ml of a solution containing 250 mM NaCl, 25 mM Tris-MOPS (morpholinopropanesulfonic acid) buffer (adjusted to the appropriate pH), ^3H_2O (4.3 μCi/ml), [^{14}C]polyethylene glycol (PEG; 0.1 μCi/ml) and a 10-fold excess of unlabeled PEG. In most experiments, 10 mM glucose was added. After incubation at 37°C for 15 min, 1-ml samples, in duplicate, were layered onto the surface of silicone oil (0.3 ml) in 1.5-ml plastic microfuge tubes and centrifuged at 12,800 × *g* for 2 min. Under these conditions, the cells pass through the silicone oil, forming a pellet at the bottom of the tube, with the aqueous phase remaining above the silicone oil (20). After samples of the aqueous phase were withdrawn for counting of radioactivity, the aqueous and silicone phases were removed by suction, the tips of the centrifuge tubes (containing the cell pellets) were cut off, and the radioactivity was counted. The ^3H counts were taken as a measure of total pellet water, and [^{14}C]PEG readings were taken as a measure of extracellular space. In most experiments, the PEG space was approximately 20% of the total pellet water. The intracellular water volume was calculated by subtracting the PEG space value from the total water space value.

Measurements of ΔpH. For quantitative measurements of ΔpH, the distribution of either ^{14}C-labeled benzoic acid (0.04 μCi/ml) or ^{14}C-labeled methylamine was determined. Cells (1.5 mg of cell protein per ml) were incubated in a solution containing 250 mM NaCl, 25 mM Tris-MOPS buffer, and 10 mM glucose. After incubation at 37°C for 10 min, 1-ml samples were layered onto the surface of silicone oil in 1.5-ml microfuge tubes and treated as described for cell water volume measurements. Intracellular and extracellular concentrations of labeled benzoic acid were determined, and the ΔpH was calculated (20).

Fluorescence measurements. The maintenance of ΔpH was estimated by monitoring the fluorescent quenching of acridine orange (4, 15, 22). Fluorescence was measured in a Perkin-Elmer spectrofluorimeter with excitation at 492 nm and emission at 530 nm. Na^+-H^+ or K^+-H^+ exchange activity was tested by imposing a ΔpH (interior acid) across the cell membrane and determining the effect of Na^+ and K^+ on the ΔpH gradient generated. A ΔpH was generated by the ammonium chloride dilution procedure described previously (4, 15). *M. gallisepticum* cells or sealed membrane vesicles were loaded with ammonium ions and diluted into ammonium-free medium. NH_4^+-loaded cells or vesicles were obtained by incubating washed cells in 0.25 M NH_4Cl containing 5 mM Tris chloride buffer (pH 8.0) and 2.5 mM $MgCl_2$ for 2 h at 37°C. Sealed membrane vesicles were loaded by including ammonium chloride (0.25 M) in the reaction mixture during the freeze-thaw/sonication cycle. The ΔpH was generated by a 100-fold dilution of the NH_4^+-loaded cells (640 μg of protein per ml) or vesicles (160 μg of protein per ml) in 2.5 ml of a solution containing 0.25 M choline chloride, 10 mM Tris hydrochloride (pH 8.0), 2.5 mM $MgCl_2$, and 5 μM acridine orange. The ΔpH was visualized from the quenching of the acridine orange signal. Na^+/H^+ and K^+/H^+ antiport activity was tested by measuring, at room temperature, fluorescence levels following the addition of 25 mM NaCl or 25 mM KCl to the reaction mixture.

$^{22}Na^+$ efflux. $^{22}Na^+$ efflux measurements were performed with preloaded *M. gallisepticum* cells and efflux initiated by the addition of glucose. Cells were preloaded with $^{22}Na^+$ by incubating cell suspensions (6 mg of cell protein per ml) for 2 h at 37°C in 0.4 M sucrose solution containing the desired concentration of NaCl, 25 mM Tris-MOPS buffer (pH 7.5), and 3.0 μCi of $^{22}Na^+$ per ml. The loaded cells were diluted 200-fold into a reaction mixture containing either 0.5 M sucrose or an isoosmotic sucrose-NaCl solution (adjusted to pH 6.5, 7.5 or 8.0), in the presence or absence of 10 mM glucose. The reaction mixtures were incubated at 32°C, and at the indicated time intervals, 10-ml portions were withdrawn and filtered through fiberglass filters (25 mm GF/C; Whatman) under negative pressure (filtration time, 3 s). The filters were washed twice with a 10-ml volume of cold 0.25 M NaCl, and the radioactivity retained on the filters was determined. Over 95% of the cells were retained on the filters, as judged by measuring the retention of [^{14}C]palmitate-labeled cells.

Materials. [7-^{14}C]benzoic acid was purchased from New England Nuclear Corp. (Boston, Mass.). [^{14}C]PEG 4000, ^3H_2O, ^{22}NaCl, and [9,10-(*m*)-^{3}H]palmitate were the products of the Radiochemical Center (Amersham, U.K.). [^{3}H]tetraphenylphosphonium (bromide salt) was purchased from the Nuclear Research Center-Negev (Beersheva, Israel). Lysine chloride, glycylglycine chloride, Tris, MES(*N*-morpholinoethanesulfonic acid), MOPS, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), dicyclohexylcarbodiimide, DNase I, bovine serum albumin (fraction V), and acridine orange were obtained from Sigma Chemical Co. (St. Louis, Mo.). Horse serum was obtained from Bio-Lab (Jerusalem, Israel). Silicone oil (550:556 grade [12:13, vol/vol]) was obtained from Dow Corning Corp. (Midland, Mich.). Diethylstilbesterol was obtained from Aldrich Chemical Co. (Milwaukee, Wis.).

RESULTS

Swelling of *M. gallisepticum* cells. The swelling of *M. gallisepticum* cells was investigated by monitoring changes in the absorbance of cell suspensions incubated in various isoosmotic salt solutions (Fig. 1). Since almost no swelling was observed in lysine chloride or glycylglycine chloride solutions, it seems that these ions are impermeable to the cells. The mild swelling in sodium isethionate and sodium glutamate and the pronounced swelling in NaCl, LiCl, and KCl solutions suggests that Na^+, K^+, and Li^+ enter the cells through either specific transport systems or nonspecific diffusion processes. The rate and extent of swelling when cells were suspended in isoosmotic sucrose-NaCl solutions increased as the ionic strength of the medium increased from 0 to 250 mM NaCl (not shown).

As the cell did not swell in the presence of glucose (Fig. 1), it seems that sodium is extruded by an energy-dependent process. If Na^+ ions were extruded by a secondary Na^+/H^+ antiport mechanism, Na^+ efflux should be driven by the proton motive force and inhibited by uncouplers which collapse $\Delta\tilde{\mu}H^+$. However, the swelling of energized *M. gallisepticum* cells suspended in an isoosmotic NaCl solution at pH 6.5 ($\Delta\tilde{\mu}H^+$ directed into the cell) was not affected by the addition of CCCP (5 μM) (Fig. 2A). Similar results were obtained over a pH range of 5.5 to 8.0 (not shown). With nonenergized cells, the rate and extent of swelling were lower in the presence of CCCP than in control untreated cells (Fig. 2A). The effect of CCCP was pH dependent and was most pronounced at the more alkaline values (pH 8.0 to 8.5). Significantly lower swelling rates and extents were also induced by CCCP (5 μM) and FCCP (2 to 10 μM) on cells suspended in isoosmotic sodium isethionate or sodium glutamate solutions. The presence of the uncouplers may allow the exit of protons as sodium ions enter the cells, negating

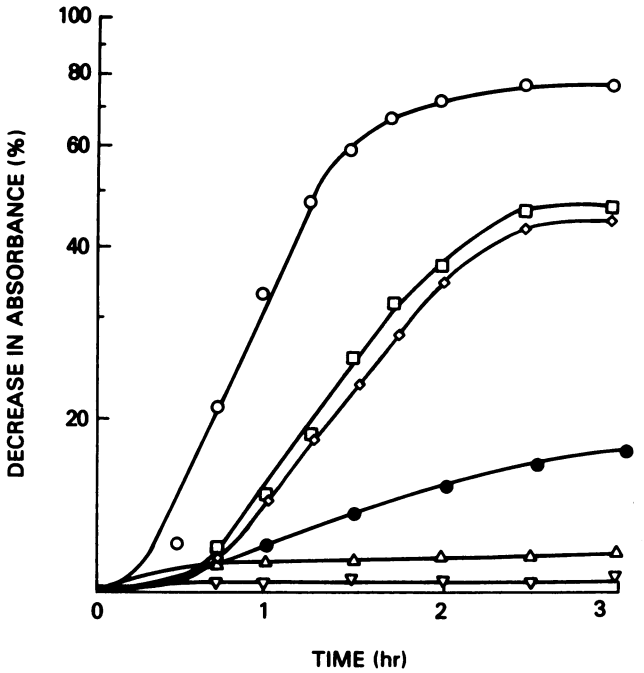

FIG. 1. Swelling of *M. gallisepticum* cells in various isoosmotic salt solutions. Cells were incubated at 37°C in the presence (solid symbols) or absence (open symbols) of 10 mM glucose in a solution containing 25 mM Tris-MOPS buffer (pH 7.5) and 225 mM sodium chloride (○, ●), sodium isethionate (□), sodium glutamate (◇), glycylglycine (△), or lysine chloride (▽). Swelling was monitored as described in Materials and Methods.

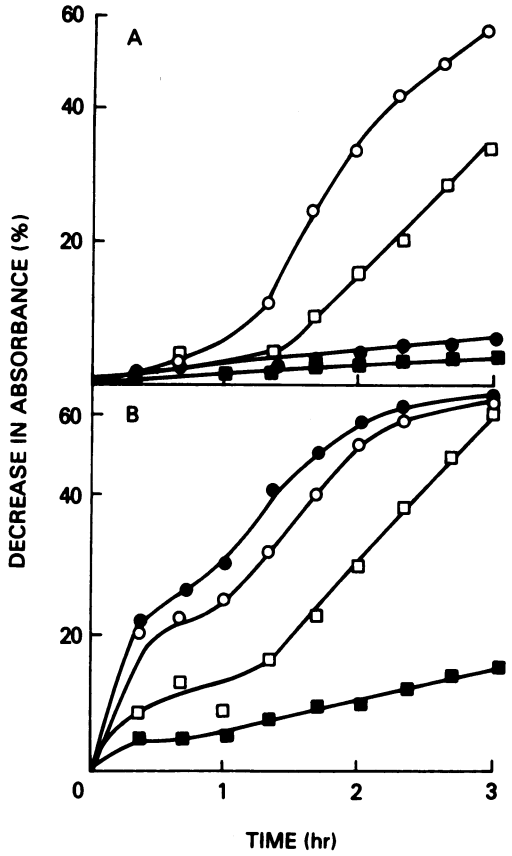

FIG. 2. Effect of CCCP on *M. gallisepticum* swelling. Swelling was performed in 225 mM NaCl (A) or KCl (B) solutions in 25 mM Tris-MOPS (pH 6.5) in the presence (solid symbols) or absence (open symbols) of 10 mM glucose, with (□, ■) or without (○, ●) CCCP.

the need for chloride to enter to maintain electroneutrality. The effect of glucose, in the presence or absence of CCCP, on the osmotic behavior of *M. gallisepticum* cells in isoosmotic LiCl solutions was similar to that observed in NaCl solutions (not shown). When cells were incubated in isoosmotic KCl, they also swelled (Fig. 2B). The addition of glucose did not protect the cells from swelling, but almost complete protection was observed when glucose and CCCP were added together (Fig. 2B).

$^{22}Na^+$ efflux from *M. gallisepticum* cells. Na^+ extrusion was studied directly by loading *M. gallisepticum* cells with $^{22}Na^+$ and following the efflux under various conditions. Figure 3 shows the kinetics of $^{22}Na^+$ efflux. In the absence of external Na^+, both the rate and extent of $^{22}Na^+$ efflux from energized cells were very low. In the presence of 250 mM NaCl, $^{22}Na^+$ efflux was glucose dependent. The glucose-dependent efflux (defined as efflux in the presence of glucose minus efflux in the absence of glucose) was rapid and linear with time over a 60-s period, until up to about 80% of the $^{22}Na^+$ was released. The rate and extent of $^{22}Na^+$ efflux in the presence of low NaCl concentrations were the same in the presence or absence of glucose (Fig. 3 and Table 1). With either energized or deenergized cells, the efflux did not have a sharp pH optimum over the pH range 6.5 to 8.0 (not shown), and efflux was resistant to inhibition by the uncouplers CCCP (10 μM) and SF6847 (1 μM). The external sodium requirement for efflux could be fulfilled by K^+ and to a lesser extent by Li^+ (Fig. 4). Under these conditions, glucose had no effect on $^{22}Na^+$ efflux. Since equilibration of the cells with sodium thiocyanate during the $^{22}Na^+$ loading procedure had no effect on efflux, a nonelectrogenic cation-sodium exchange mechanism is suggested.

To test this exchange further, the intracellular Na^+ concentration was varied from 23 μM to 23 mM while an external Na^+ concentration of 125 mM was maintained. This resulted in a linear initial rate of efflux which was saturated at the maximum intracellular sodium concentration (23 mM) (not shown). Lineweaver-Burk analysis showed that the exchange in an isoosmotic efflux medium containing 125 mM NaCl and 250 mM sucrose in 10 mM Tris-MES buffer, pH 7.5, had a K_m of 29 mM and a V_{max} of 4 μmol/g of cell protein per min.

The characteristics of energy-independent $^{22}Na^+$ efflux under conditions in which no sodium gradient existed ($[Na^+_{in}] = [Na^+_{out}]$) were similar to those described above for uphill sodium efflux ($[Na^+_{in}] < [Na^+_{out}]$). The efflux showed the same pH profile, resistance to inhibition by uncouplers, and ability for exchange in the presence of KCl or LiCl in the efflux medium (not shown).

The effect of uncouplers on glucose-dependent $^{22}Na^+$ efflux was studied in cells that were suspended in isoosmotic NaCl suspension medium (250 mM NaCl) and adjusted to various pHs over the range 6.0 to 8.0. The uncoupler SF6847 (0.5 μM) did not inhibit $^{22}Na^+$ efflux over the pH range tested (Fig. 5). The enhancement of $^{22}Na^+$ efflux at pH 6.5 by SF6847 is consistent with the premise that Na^+ is extruded electrogenically. Under conditions in which the membrane is permeabilized by uncouplers, H^+ can be taken up in response to the generated $\Delta\psi$ (negative inside), reducing the inhibitory effect of this gradient on cation extrusion.

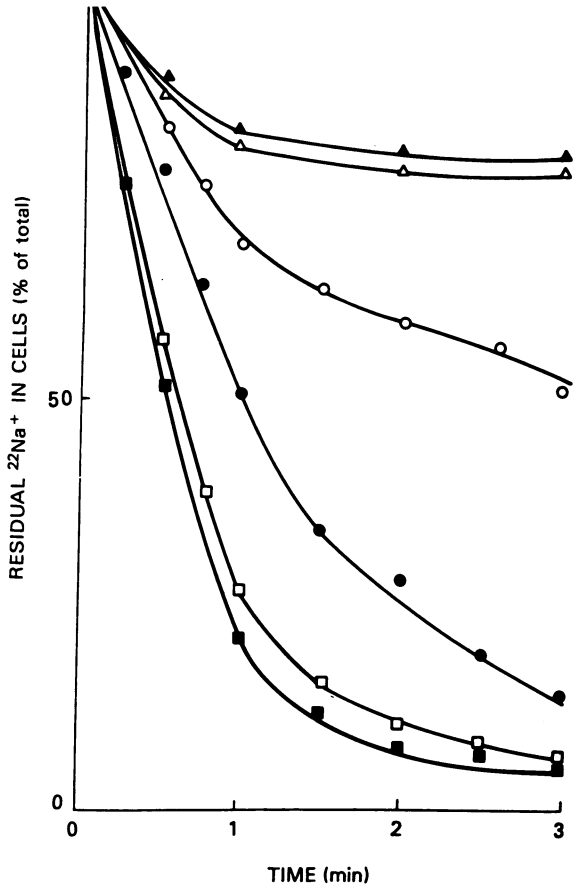

FIG. 3. Effect of sodium and glucose on $^{22}Na^+$ efflux. Cells were equilibrated with 25 mM $^{22}Na^+$ and transferred to various isoosmotic sucrose-NaCl solutions with (solid symbols) or without (open symbols) 10 mM glucose. Symbols: △, ▲, 0.5 M sucrose; □, ■, 0.5 M sucrose plus 1 mM NaCl; ○, ●, 0.25 M NaCl.

Effect of a transmembrane pH gradient on Na^+ uptake. To study further whether the driving force for the energy-dependent sodium transport is a proton electrochemical gradient, an imposed ΔpH was generated in intact cells or sealed membrane vesicles by equilibrating the cell or membrane preparations with NH_4Cl and diluting them into a buffer containing 250 mM choline chloride, adjusted to pH 8.0. The ΔpH was produced by the dissociation of NH_4^+ to NH_3 and H^+ and the downhill diffusion of NH_3 out of the cells. The ΔpH was visualized from the quenching of the weak base acridine orange. The addition of either NaCl (25 mM) or KCl (25 mM) to cells or the sealed membrane vesicle preparation, after a substantial ΔpH was generated, did not affect the fluorescence intensities (Fig. 6). As expected, nigericin induced rapid dequenching. These results suggest that under the experimental conditions described (low intracellular pH, high NH_4^+ concentrations), there is no detectable Na^+/H^+ antiport activity.

TABLE 1. Effect of NaCl on $^{22}Na^+$ efflux[a]

NaCl (mM)	Glucose (10 mM)	$^{22}Na^+$ efflux (kcpm/min)	% of initial $^{22}Na^+$ content remaining
0	+	0.67	79
	−	0.67	77
1	+	3.20	6
	−	3.15	7
100	+	2.35	6
	−	2.30	7
150	+	1.55	8
	−	1.50	13
200	+	1.40	10
	−	ND[b]	21
250	+	1.40	14
	−	1.15	51

[a] $^{22}Na^+$ efflux was assayed at pH 7.5 as described in Materials and Methods, and results are presented as initial rates $^{22}Na^+$ content was determined after 3 min of incubation.
[b] ND, Not determined.

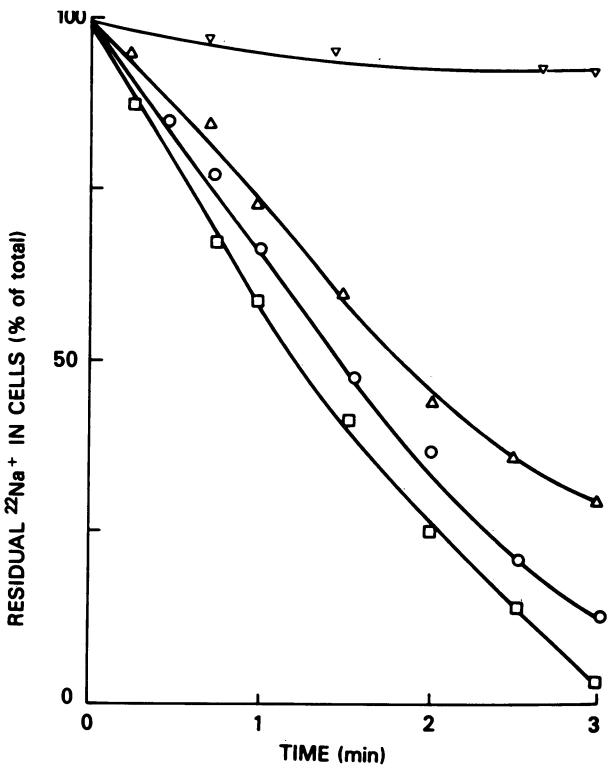

FIG. 4. $^{22}Na^+$ efflux from deenergized cells in the presence of various monovalent cations. Washed cells were loaded with $^{22}Na^+$ and depleted of an energy source as described in Materials and Methods. The efflux medium contained an isoosmotic solution of sucrose (▽) or sucrose plus 25 mM KCl (□), NaCl (○), or LiCl (△).

Effect of CCCP on growth of *M. gallisepticum* in high-salt media. A typical growth curve for *M. gallisepticum* in Edward medium containing 124 mM NaCl and 4% horse serum is presented in Fig. 7. The increase in absorbance was accompanied by a decrease in the pH of the medium from pH 7.8 at the early exponential phase of growth to pH 6.0 at the late exponential phase of growth (Fig. 7). When CCCP was added to the growth medium at the early exponential phase of growth, cells continued to grow until the pH of the external medium approached 7.1, at which point growth ceased. When the pH of the external medium was raised to pH 7.6, growth resumed. The membranes of *M. gallisepticum* were completely permeable to protons at the concentration of CCCP used (100 μM) (not shown).

DISCUSSION

Mycoplasmas lack a rigid cell wall, are bound by a single membrane, and are generally susceptible to osmotic lysis (3,

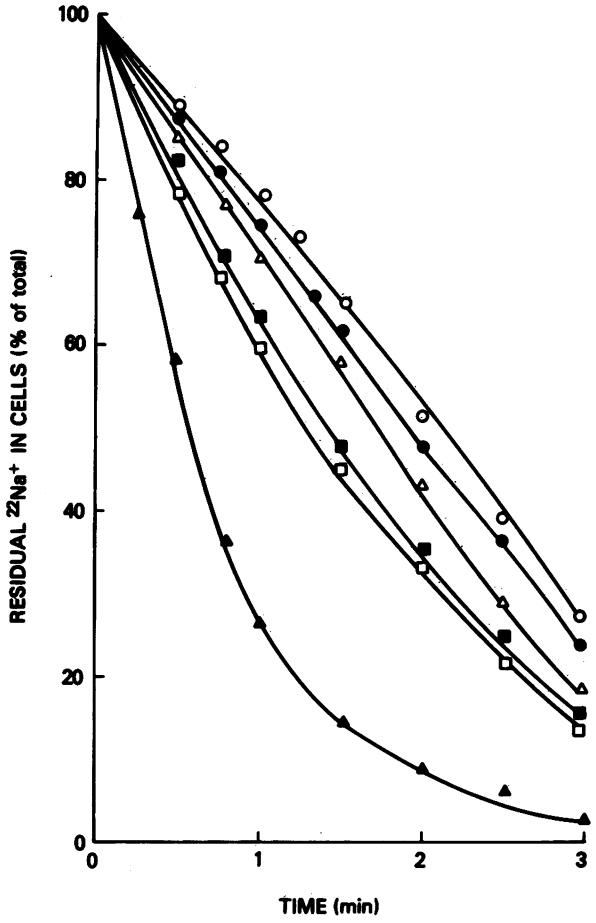

FIG. 5. Effect of pH and uncouplers on $^{22}Na^+$ efflux. Efflux was carried out in medium with 250 mM NaCl containing 10 mM glucose in the presence (solid symbols) or absence (open symbols) of 0.5 μM SF6847 at pH 8.0 (○, ●), pH 7.5 (□, ■), or pH 6.5 (△, ▲).

17, 18). Thus, it has been suggested that they act as slightly imperfect osmometers (17, 18, 21). When *M. gallisepticum* cells are incubated in an isoosmotic NaCl medium of high ionic strength in the absence of an energy source, they swell (20). Swelling was suggested to be due to the inward diffusion of Na^+ and Cl^- (and water) into the cell as a result of Gibbs-Donnan forces (generated by intracellular anionic macromolecules) (8, 20). Our results, which show no swelling in isoosmotic solutions of lysine chloride or glycylglycine chloride, mild swelling in sodium isethionate or sodium glutamate, and pronounced swelling in NaCl support this view. Chloride apparently acts as an osmotic counterion to maintain electroneutrality. Volume regulation therefore reflects a balance between active cation and passive anion movements across the membrane. It also depends on sufficient Na^+ extrusion to compensate for the colloid osmotic pressure of intracellular macromolecules and Na^+ that has diffused into the cell. A major question is how is Na^+ extruded?

Recent studies have proposed that *M. gallisepticum* extrudes Na^+ by a combination of a proton-translocating ATPase and an Na^+/H^+ antiporter (8–10). The authors, however, were not able to explain why uncouplers of $\Delta\tilde{\mu}H^+$ failed to cause swelling. Furthermore, no direct correlation between sodium movements across the membrane and $\Delta\tilde{\mu}H^+$ was shown. In the present study, possible mechanisms for sodium extrusion were reexamined, beginning with possible Na^+ extrusion by an Na^+/H^+ antiporter. The results indicate that no active Na^+/H^+ antiport activity is detectable in *M. gallisepticum* cells. This conclusion is based on the following evidence. (i) No inhibition of $^{22}Na^+$ efflux was observed in either uphill or downhill transport after treatment of the cells with CCCP (Fig. 5). (ii) The swelling of energized cells suspended in isoosmotic NaCl was not enhanced by CCCP (Fig. 2). (iii) No Na^+-H^+ exchange was observed in cells or sealed membrane vesicles after imposition of a pH gradient (acidic inside) by the ammonium chloride dilution procedure (Fig. 6) (23). Similarly, generation of a ΔpH by the same procedure did not drive $^{22}Na^+$ uptake in sealed membrane vesicles (data not

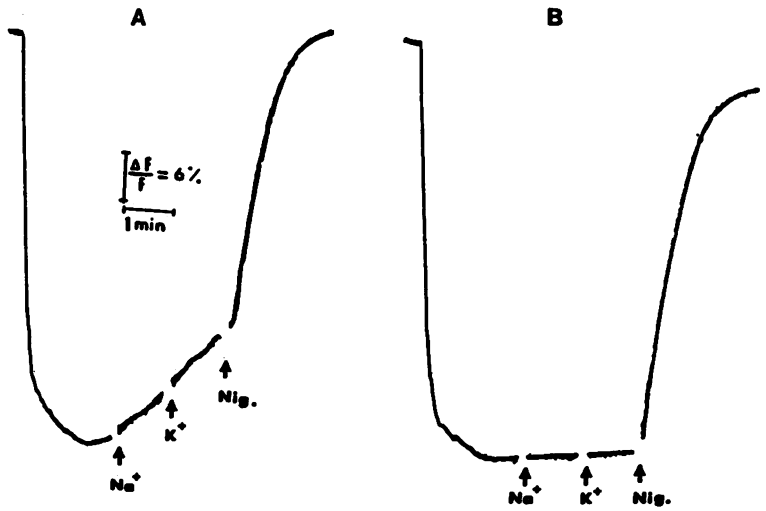

FIG. 6. Effect of Na^+ and K^+ on an imposed ΔpH in cells and sealed membrane vesicles. Cells or sealed membrane vesicles loaded with 0.25 M NH_4Cl were diluted into a fluorometer cuvette containing 0.25 M choline chloride and acridine orange, and fluorescence was measured as described in Materials and Methods. At the times indicated by arrows, NaCl (25 mM), KCl (25 mM), and nigericin (Nig., 1 μM) were added. (A) Intact cells; (B) sealed membrane vesicles.

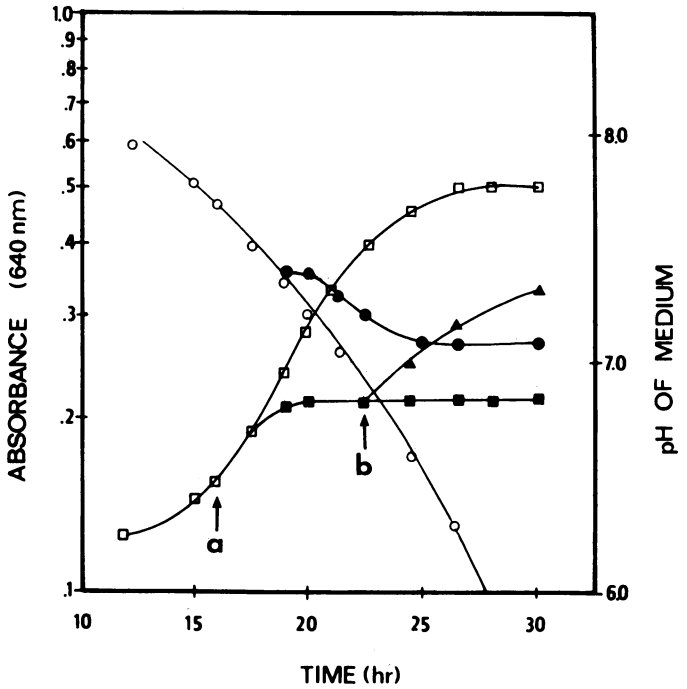

FIG. 7. Effect of CCCP on growth of *M. gallisepticum*. Growth was monitored in Edward medium (19) containing 4% horse serum in the absence (open symbols) or presence (solid symbols) of CCCP (100 μM). The A_{640} of the culture (□, ■) and pH of the culture (○, ●) were recorded. At time a, CCCP (100 μM) was added to part of the growth medium (●). At time b, the pH of the part of the growth medium containing CCCP was adjusted to pH 7.6 and the A_{640} was recorded (▲).

shown), but did drive $^{22}Na^+$ uptake in *Escherichia coli* reconstituted membrane vesicles (23). (iv) Under low-stringency conditions, in which approximately 50% homology could be detected, no hybridization between plasmid pGM36 containing the conserved *E. coli* Na^+/H^+ antiporter gene (4) and a *Hin*dIII digest of *M. gallisepticum* genomic DNA was observed (M. Shirvan, unpublished data). (v) Cells were able to grow and remain viable in a high-salt-containing medium (124 mM NaCl), in the presence of CCCP, over the test period (16 h) (Fig. 7). Since volume is actively regulated in mycoplasmas, the primary extrusion of an ion other than H^+ would be expected to play a central role.

These observations strongly suggest that Na^+ movement is neither driven by $\Delta\tilde{\mu}H^+$ nor inhibited by a collapse of $\Delta\tilde{\mu}H^+$, the proposed driving force. Taken as a whole, the data presented in this work question the conclusion (26) that the Na^+/H^+ antiporter is ubiquitous in bacteria. As Na^+/H^+ antiport activity is not required under physiological growth conditions, a primary mechanism for sodium extrusion is suggested.

The presence of an outward-directed Na^+ pump could act to maintain the extracellular/intracellular Na^+ ratio higher than expected by the Donnan distribution. The effect would be to osmotically balance the colloid osmotic effect and work against osmotic lysis. Volume regulation in *M. gallisepticum* would therefore depend on primary sodium extrusion. The lack of a cell wall would require cells to have a regulated system to be able to adapt to a wide range of environments. Yet the primitive nature of mycoplasmas dictates that this mechanism be simple. A process can be considered whereby suspending cells in an isoosmotic solution of permeable cations (such as the natural habitat of mycoplasmas) would result in the diffusion of these cations into the cell, increasing cellular ionic strength. This increase would subsequently stimulate the sodium pump to extrude Na^+ and reduce the intracellular osmotic pressure. Glucose enhancement of $^{22}Na^+$ efflux in high-ionic-strength solutions, and the proposal (24) that cell ionic strength is involved in the volume regulatory mechanism of *E. coli*, support this model.

ACKNOWLEDGMENT

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