JOURNAL OF BACTERIOLOGY, Sept. 1985, p. 1243–1249
0021-9193/85/091243-07$02.00/0

Vol. 163, No. 3

Cell Volume Regulation in *Mycoplasma gallisepticum*

CAREY LINKER† AND T. HASTINGS WILSON*

Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts 02115

Received 28 January 1985/Accepted 9 June 1985

Mycoplasma gallisepticum **cells incubated in 250 mM NaCl solutions in the absence of glucose showed a progressive fall in intracellular ATP concentration over a period of 2 to 3 h. When the ATP level fell below 40 μM the cell began to swell and become progressively permeable to [^{14}C]inulin and leak intracellular protein and nucleotides. The addition of nondiffusable substances such as $MgSO_4$ or disaccharides prevented swelling, suggesting that NaCl (and water) entry was due to Gibbs-Donnan forces. The addition of glucose after the initiation of cell swelling increased intracellular ATP, induced cell shrinkage, and prevented the release of intracellular components. The ATPase inhibitor dicyclohexylcarbodiimide, which collapsed the chemical and electrical components of the proton motive force, caused rapid cell swelling in the presence of glucose (and high intracellular ATP levels). Extracellular impermeable solutes such as $MgSO_4$ and disaccharides prevented swelling of dicyclohexylcarbodiimide-treated cells incubated in NaCl. It was postulated that Na^+ that diffused into the cell was extruded by an electrogenic Na^+-H^+ exchange (antiport) energized by the proton motive force established by the dicyclohexylcarbodiimide-sensitive H^+-ATPase.**

All cells are faced with the problem of volume regulation even when they are suspended in a medium that is isosmotic with the internal environment. The osmotic problem is caused by the presence of intracellular nondiffusable charged macromolecules. The resulting Gibbs-Donnan effects give rise to the entry of salts and water, inducing swelling and eventual lysis of the cell if there is no opposing reaction (11, 35). This swelling phenomenon, referred to as colloid osmotic swelling by Wilbrandt (34) and later studied by Jacobs and Stewart (9), has been reviewed by Leaf (12), Tosteson (32), and Macknight and Leaf (17, 18). Two biological solutions have been devised to prevent the tendency of cells to swell due to this colloid osmotic effect: (i) a rigid cell wall to prevent an increase in volume; and (ii) ion pumps which extrude ions (and water) that enter by diffusion. Examples of the cell wall solution to the osmotic problem are found in most bacteria and plants. In other cells the ionic pumps extrude salt and water to regulate cell volume. Such ion pumping mechanisms of volume regulation have been suggested in a variety of animal cells (17, 18). The use of the Na^+,K^+-ATPase of animal cells for this purpose has been extensively investigated (17, 18, 32, 33).

Most bacteria possess rigid cell walls and are generally assumed to require no additional defenses against swelling. In fact, *Escherichia coli* maintains a greater internal osmolarity than the external medium resulting in turgor pressure, which is necessary for normal growth and cell division (7). Organisms of the cell wall-less class *Mollicutes* (including the families *Mycoplasmataceae* and *Acholeplasmataceae*) are, however, vulnerable to osmotic stress. It has long been known that these organisms tend to lose intracellular components and lyse after prolonged incubation at 37°C in the absence of an energy source (31). Energy-dependent ion pumps are implicated in volume regulation of *Acholeplasma laidlawii* (10) and *Mycoplasma gallisepticum* (27). Jinks et al. (10) described an Na^+-stimulated ATPase in *A. laidlawii* and postulated that it acted to regulate cell volume by extruding sodium. Benyoucef et al. (3) described, in *Mycoplasma mycoides* var. Capri, energy-dependent Na^+ extrusion against a concentration gradient that was dependent on external K^+. They inferred from this and other data the existence of an ATP-consuming Na^+-K^+ exchange system in addition to a Na^+-H^+ antiporter and H^+-pumping ATPase.

It is the purpose of this investigation to study the factors involved in the volume regulation of *M. gallisepticum*. This organism possesses a dicyclohexylcarbodiimide (DCCD)-sensitive ATPase that is believed to function as an electrogenic proton pump (16) and generate an electrochemical gradient of protons across the membrane. Indeed, a proton motive force of 60 to 130 mV (inside negative and alkaline) has been measured in energized cells (27). In this paper we show that energy-depleted cells swell, over a period of hours, when exposed to NaCl. The addition of glucose prevents or reverses the swelling. DCCD causes rapid swelling even in the presence of high intracellular ATP. DCCD-induced swelling does not occur in the presence of extracellular impermeable solutes such as $MgSO_4$ or sucrose. Either a pH gradient (inside alkaline) or an electrical gradient (inside negative) can maintain cell volume. Possible ionic mechanisms for volume regulation are discussed.

MATERIALS AND METHODS

Growth of the organisms. *M. gallisepticum* (strain A5969) was grown in Edward medium (26), containing 4.7% horse serum (Difco Laboratories). The medium was inoculated with 1% of an overnight culture and incubated at 37°C for 18 to 25 h without aeration. Growth was monitored by measuring the pH of the culture and the absorbance at 640 nm. Cells were harvested at an optical density (640 nm) of 0.20 to 0.28 (pH 6.6 to 7.0). Routine examination of cultures by phase-contrast microscopy insured that they were free of other bacteria as contaminants. Cells were harvested by cooling the culture in an ice-water bath for 10 min followed by centrifugation at 16,000 × *g* for 20 min at 4°C. The cells were suspended in an appropriate washing solution at 4°C by gentle vortexing followed by centrifugation at 14,000 × *g* for 15 min at 4°C. The pellet was suspended in a small volume (5 ml or less) of an appropriate solution and kept on ice for 10 min to allow any cell clumps to settle out. The clump-free portion of the cell

* Corresponding author.
† Present address: Stanford University, Stanford, CA 94305.

suspension was then appropriately diluted and kept on ice for later use.

Measurement of cell water. Cells at a final concentration of 1 to 4 mg of cell protein per ml were incubated in 2.3 ml of a buffered solution containing ^3H_2O (4.3 μCi/ml) and [^{14}C]inulin (0.4 μCi/ml; 100 μg/ml). Glucose (5 to 10 mM) was added if necessary. After incubation at 37°C for 5 to 15 min, 1-ml duplicate samples were applied on top of 0.5 ml of silicon oil (Contour XF-1792B) in a 1.5-ml plastic microfuge tube and centrifuged at 12,800 × *g* for 1 to 2 min. Under these conditions, the cells pass through the oil and form a pellet at the bottom of the tube. The aqueous phase remained above the oil. Samples of the supernatant fluid were saved for determination of ^{3}H and ^{14}C. Both the aqueous phase and oil were removed by suction, and the bottom of the plastic centrifuge tube (containing the pellet) was cut off with a razor blade and placed in a vial containing 10 ml of scintillation fluid (20). With vigorous vortexing the cell pellet was freed from the plastic tip and broken into small fragments. The total water space minus the inulin space was taken as the intracellular water space of the cell pellet under conditions where inulin (molecular weight, 5,000) did not penetrate the cell.

Volume measurement by changes in optical density. Cell volume was monitored in some experiments by measurement of the optical density of cell suspensions with a Gilford or Klett-Summerson spectrophotometer.

ATP measurement. Cells (0.4 ml) were added to a test tube containing 0.1 ml of 3 M perchloric acid. After 10 min at 0°C, 0.3 ml of 1 M KOH was added, and the potassium perchlorate was allowed to settle. Samples were taken, and the ATP level was determined by the luciferase method of Cole et al. (5).

Release of intracellular components. Several 1-ml samples of cells were pipetted onto the surface of 0.5 ml of silicon oil in 1.5-ml plastic microfuge tubes and centrifuged at 12,800 × *g* for 1 min. The supernatants were then centrifuged at 45,000 rpm for 30 min at 4°C in a 50 Ti rotor. The protein content of the ultracentrifugation supernatant was determined by the method of Peterson (21). The absorbance of this supernatant at 260 nm was also determined. The release of components is expressed as a percentage of the values obtained from cells completely lysed by incubation at 37°C with either 250 μM DCCD or 10 μM gramicidin for 40 min.

Protection against DCCD-induced lysis by sugars. Cells were washed in 200 mM NaCl–10 mM 3-(*N*-morpholine)propanesulfonic acid–10 mM glucose and suspended in the same solution at approximately 33 mg of cell protein per ml. Stock cells (0.1 ml) were added to 5 ml of a 400 mM solution of the indicated sugars or 200 mM NaCl (control) plus 9 mM glucose, and the optical density was determined (640 nm). DCCD was added to give a final concentration of 200 μM. After a 12-min incubation at 37°C, a second optical density reading was taken. Cells incubated in 200 mM NaCl plus 9 mM glucose (with no DCCD) showed a final optical density of 91% of the initial value. Because of the high refractive index of 0.4 M solutions of di- and trisaccharides, the absolute readings of cells in these solutions are lower than readings of cells in NaCl solutions. For this reason the data are expressed as the percent change from initial values.

Measurement of ΔpH and membrane potential. The ΔpH was measured by the benzoic acid technique (19). The membrane potential was measured with radioactive tetraphenylphosphonium (27).

Materials. ^3H_2O, [^{14}C]inulin, $^{86}Rb^+$, [^{14}C]benzoic acid, and [^{14}C]lactose were from New England Nuclear Corp. DCCD, carbonyl cyanide-*p*-trifluoro-methoxyphenyl hydrazone, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, *N*-2-acetamido-2-aminoethanesulfonic acid, and *N*,*N*-bis(2-hydroxymethyl)-2-aminoethanesulfonic acid were from Sigma Chemical Co. Carbonyl cyanide-*m*-chlorophenyl hydrazone, nigericin, valinomycin, gramicidin were from Calbiochem. [^{3}H]tetraphenylphosphonium was a generous gift of Ronald Kaback. [^{14}C]inulin was purified by either paper chromatography or dialysis to remove traces of fructose. DCCD, carbonyl cyanide-*m*-chlorophenyl hydrazone, carbonyl cyanide-*p*-trifluoro-methoxyphenyl hydrazone, nigericin, valinomycin, and gramicidin stocks were prepared in ethanol.

RESULTS

Volume changes in response to external osmolarity. Whereas most *Mollicutes* behave as nearly perfect osmometers and lyse rapidly in media of low ionic strength (6, 24), *M. gallisepticum* is somewhat resistant to osmotic

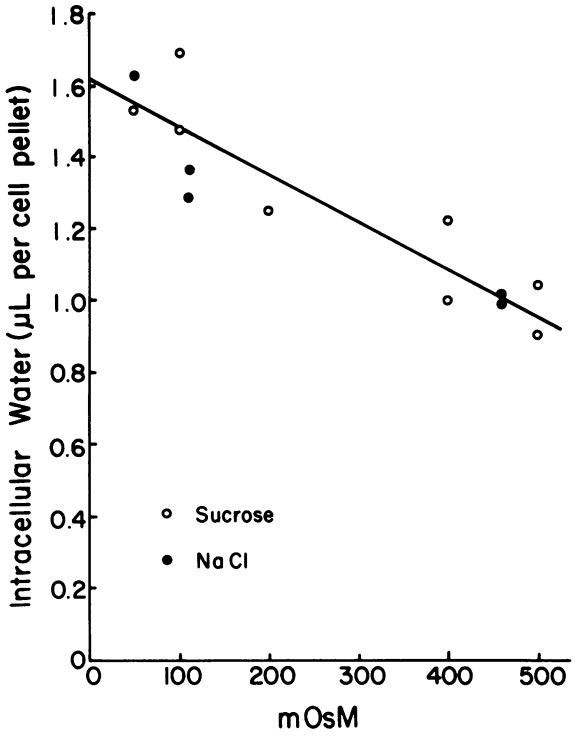

FIG. 1. Cell volume versus osmolarity of medium. One liter of cells was grown to an optical density at 640 nm of 0.25 (pH 6.9). One half of the cells was washed once in 250 mM NaCl–10 mM morpholinepropanesulfonic acid–10 mM glucose (pH 7.0) and suspended in 2 ml of the same buffer (●). The other half was washed once in 400 mM sucrose–10 mM morpholinepropanesulfonic acid–10 mM glucose (pH 7.0) and suspended in 2 ml of the same sucrose buffer (○). The stock cell concentration was 45 mg of cell protein per ml. Samples of stock cells (30 μl) were added to 3.0 ml containing ^3H_2O, [^{14}C]inulin, 10 mM morpholinepropanesulfonic acid–10 mM glucose (pH 7.0) and various concentrations of either NaCl or sucrose. These were incubated at 37°C for 5 min. Two 1.0-ml samples were removed, and cell water was determined as described in the text. It was necessary to use different density silicone oils for the various sucrose solutions. The data presented were derived from two separate experiments.

lysis (24, 25). Rottem and Verkleij (28) have demonstrated the presence of unusual segregated lipid domains in the membrane of this species which correlate with resistance to lysis. They suggested that the interface between protein-free and protein-rich membrane patches becomes ion permeable and that loss of intracellular solute imparts resistance to cell swelling.

M. gallisepticum cells suspended in either NaCl or sucrose at 37°C changed their cell volume as the medium osmolarity was varied, although they did not behave as perfect osmometers (Fig. 1). At even the lowest osmolarity (approximately 50 mosM), the cells remained impermeable to inulin (data not shown). At osmolarities less than 50 mosM the cells failed to pellet through silicon oil; thus measurement of the intracellular water was not possible by this technique.

Release of intracellular components during swelling. When cells were incubated in 250 mM NaCl at 37°C in the presence of 1 mM glucose the cell volume, as measured by optical density, remained constant for 2 to 3 h (Fig. 2A). Thereafter there was a continuous decrease in optical density (cell swelling). Figure 2A demonstrates that during this period of swelling there was a progressive release of intracellular protein and UV-absorbing compounds into the incubation medium. Even after 1,500 min of incubation, cells could still be centrifuged through silicon oil to form a pellet, indicating the retention of some structural integrity. The addition of 50 mM glucose induced initial cell shrinkage, followed by return to the original volume for a period of 4 h with little loss of intracellular components (Fig. 2A). When 10 mM glucose was added after the initiation of swelling (Fig. 2B), the cells reduced their volume and retained their components.

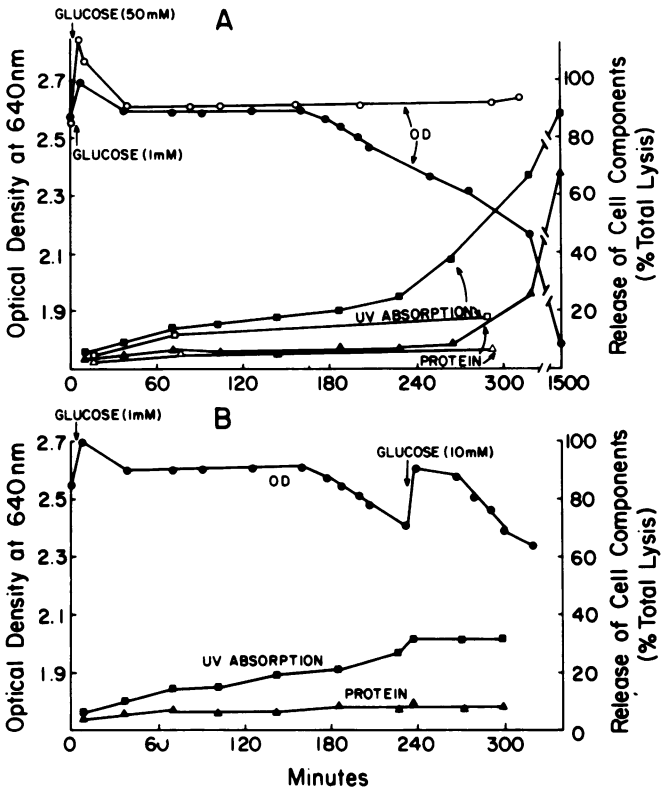

FIG. 2. Release of intracellular components during swelling. Midexponential-phase cells (optical density at 640 nm of 0.25, pH 6.8) were washed once in 250 mM NaCl–10 mM morpholinepropanesulfonic acid (pH 7.0) and suspended in 60 ml of the same solution (3 mg of cell protein per ml, optical density of 2.55). (A) The stock cell suspension (40 ml) was incubated at 37°C, and glucose (1 mM) was added where indicated by the arrow (●, ■, ▲). To 10 ml of stock cells, 50 mM glucose (○, □, △) was added at the arrow. (B) To a 10-ml sample of stock cells 1 mM glucose was added at 5 min (first arrow), and 10 mM glucose was added at 235 min (second arrow). Swelling was followed by the optical density of the cell suspensions at 640 nm (●). The release of intracellular protein (▲) and UV (260 nm)-absorbing molecules (■) into the incubation medium is expressed relative to cells incubated with 250 μM DCCD (100% lysis).

Correlation of optical density, total water, inulin space, and ATP during swelling. Since glucose prevented swelling and lysis, it was of interest to correlate these changes in volume with the intracellular ATP concentration. *M. gallisepticum* converts glucose to lactic acid and generates ATP by substrate-level phosphorylation. Cytochromes are absent in these cells, and oxidative phosphorylation is not known to occur (22).

In the next experiment (Fig. 3), ATP and cell volume measurements were made in cells incubated in NaCl for various periods of time. Cells were exposed to 250 mM NaCl at 37°C, and swelling was followed by measurement of the optical density and by determination of total pellet water (^3H_2O). The addition of 1 mM glucose (Fig. 3A) at 14 min induced a rapid increase in cellular ATP (maximum of 1.2 mM) and cell shrinkage as indicated by the rise in optical density and the fall in intracellular water. The cells then maintained their volume for the next 2 to 3 h. When the intracellular ATP fell to 40 μM (120 min), swelling began to occur, as indicated by an increase in intracellular water. The optical density began to fall about 30 min later. Thereafter both optical density and cell water measurements indicated continued swelling. After the first phase of swelling (160 min), the cells became permeable to inulin, as indicated by an increase in the percentage of the total water space that became accessible to inulin (without any significant increase in the size of the pellet).

In a parallel experiment (Fig. 3B), 10 mM glucose was added at the initiation of swelling (190 min), inducing an immediate reduction in cell volume as measured by both techniques concomitant with an increase in the intracellular ATP concentration. In addition, the normal impermeability of the cells to inulin was regained. A similar energy-dependent reversal of the permeability properties of the cells was obtained with [^{14}C]lactose instead of [^{14}C]inulin.

Swelling induced by the ATPase inhibitor DCCD. If the ATPase known to be present in these cells (16) is an ion-translocating mechanism involved in volume regulation, then DCCD, an inhibitor of ATPase (16), should stimulate cell swelling even in the presence of glucose and high ATP levels. DCCD induced a concentration-dependent swelling of cells suspended in NaCl at 37°C (Fig. 4). At a concentration of 50 μM, DCCD significantly stimulated swelling, whereas 150 to 200 μM produced a maximal effect. The addition of 10 mM glucose failed to affect the rate of DCCD-induced cell swelling (data not shown). DCCD did not affect the rate or extent of ATP synthesis in the presence of glucose. Thus DCCD-induced swelling did, indeed, occur in the presence of high levels of ATP. DCCD dissipated both the chemical and electrical components of the proton motive force, as would be expected for an inhibitor of the electrogenic proton pumping ATPase. This was true for cells incubated in NaCl and glucose (Table 1) or sucrose plus

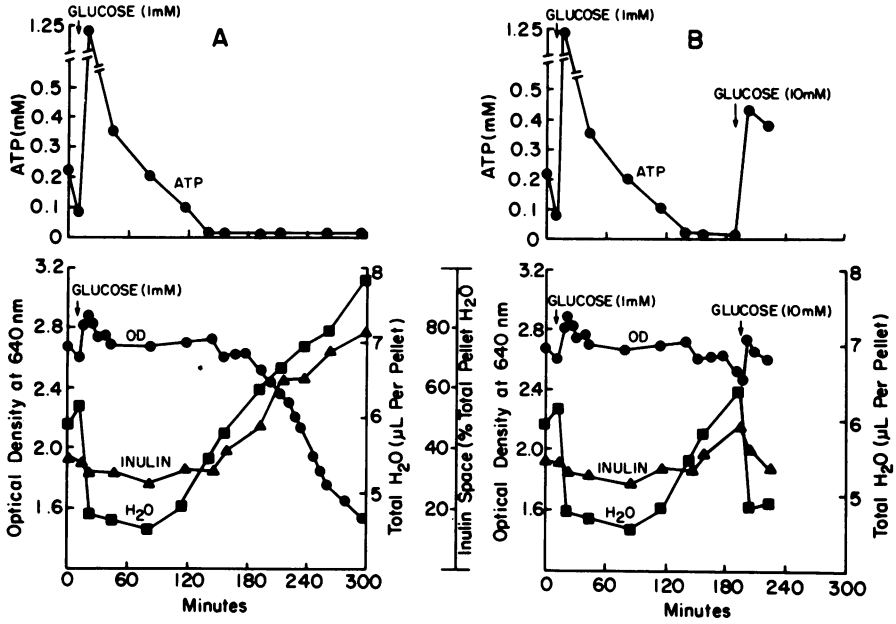

FIG. 3. Correlation of optical density, total water, inulin space, and ATP during swelling. Midexponential-phase cells (optical density at 640 nm of 0.23, pH 7.0) were washed once in 250 mM NaCl–10 mM morpholinepropanesulfonic acid (pH 7.0) and suspended in the same buffer at 3.5 mg of cell protein per ml (optical density of 2.66). (A) Glucose (1 mM) was added to 42 ml of stock cell suspension at 37°C at the arrow. (B) Glucose (10 mM) was added to a 5-ml portion of the above cells at the second arrow. Upper graphs display the intracellular content of ATP. Lower graphs present the optical density determinations at 640 nm (●), the total ^3H_2O spaces of pellets from 2-ml samples of the cell suspension (■), and the percentages of this space accessible to [^{14}C]inulin (▲).

glucose (data not shown). Therefore, regulation of cellular volume appears to require the presence of an electrochemical potential difference of protons across the membrane.

Inhibition of swelling by impermeable solutes. If the intracellular impermeant molecules were responsible for the swelling of energy-depleted cells suspended in NaCl solutions, then the addition of impermeant molecules to the external medium should prevent this swelling. Cells incubated in 0.5 M sucrose failed to swell even after the ATP concentration fell to undetectable levels (Fig. 5). An experiment was performed to ascertain the concentration of sucrose necessary to prevent DCCD-induced swelling. Cells were incubated in NaCl plus DCCD in different combinations of sucrose and NaCl such that the external osmolarity remained constant. At a concentration of 400 mM, sucrose completely prevented swelling, whereas at concentrations as low as 100 mM the disaccharide inhibited the rate of swelling. Lactose, maltose, melibiose, raffinose, and maltotriose

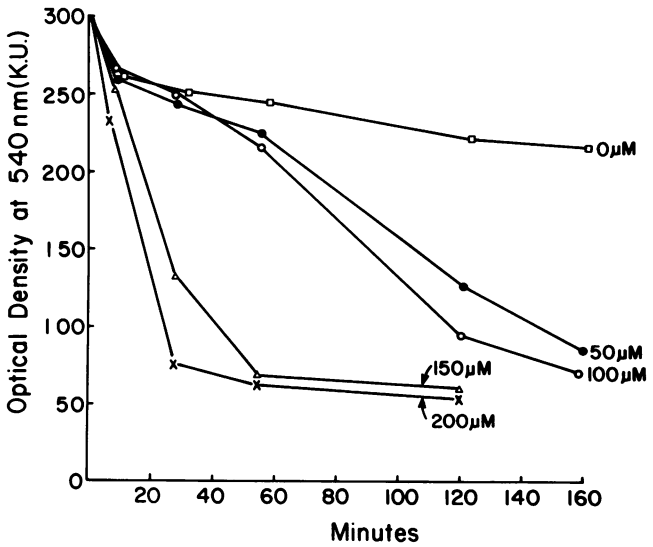

FIG. 4. Effect of DCCD on cell swelling. Cells were grown to an optical density (640 nm) of 0.29 (pH 6.2), washed once in 0.3 M $MgSO_4$, and suspended in 3 ml of the same medium. A sample (0.12 ml) of these cells was diluted into a final volume of 5.0 ml (0.84 mg of cell protein per ml) containing 191 mM NaCl–10 mM morpholinepropanesulfonic acid (pH 7.0) and incubated at 37°C in the following concentrations of DCCD: 0 µM (□), 50 µM (●), 100 µM (○), 150 µM (△), and 200 µM (×). Swelling was followed at 540 nm (Klett units).

TABLE 1. Effect of DCCD and nigericin on proton motive force

Expt.	Inhibitor (µM)	ΔΨ (mV)	ΔpH (mV)	$\Delta\bar{\mu}_{H^+}$ (mV)
1[a]	None	44	13	57
	DCCD (100)	11	5	16
2[a]	None	49	23	72
	DCCD (100)	19	0	19
3[b]	None	39	40	79
	Nigericin (15)	37	14	51
4[b]	None	32	79	111
	Nigericin (15)	45	14	59

[a] Cells (2 ml) at a concentration of 2.5 mg of cell protein per ml were incubated at 37°C for 5 min in 144 mM NaCl–150 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid–11 mM glucose with or without 100 µM DCCD. ΔΨ, ΔpH, and cell water were determined as described in the text.

[b] Cells (2 ml) were incubated for 10 min at 37°C in 500 mM sucrose–50 mM *N*-2-acetamido-2-aminoethanesulfonic acid (pH 6.5)–4.3 mM KCl with or without 15 µM nigericin.

show the same protective effect as sucrose. Energy-depleted cells incubated in $MgSO_4$ (300 mM) or polyethylene glycol 400 (470 mM) also failed to swell at 37°C (data not shown).

The fact that DCCD failed to induce swelling in the presence of impermeable molecules provides evidence that this inhibitor does not compromise membrane integrity. Furthermore, DCCD did not affect substrate level phosphorylation in the presence of these impermeable molecules (data not shown).

Effect of the anion on swelling. Cells were washed and suspended in 500 mosM sodium phosphate (192 mM), NaCl (250 mM), or Na_2SO_4 (167 mM) at pH 7.0 with and without glucose. In the presence of sodium phosphate or Na_2SO_4, there was very little swelling with or without glucose at 37°C. In the presence of NaCl, swelling followed by lysis occurred after 150 min; this was prevented by glucose (10 mM). The addition of DCCD (300 μM) caused very rapid swelling and lysis of cells suspended in NaCl, but much slower swelling with the other two salts.

Effect of external pH on swelling. Cells swelled much more rapidly at pH 8.0 than at 6.5 or 5.5 in 250 mM NaCl without glucose. At all three pH values, glucose prevented swelling. Sucrose prevented swelling at pH 5.5 and 6.5, but not at pH 8.0, perhaps because cells become permeable to sucrose at that higher pH. Similar pH-dependent swelling in this species was described by Shirvan et al. (29).

Effect of temperature on swelling. Cells exposed to 250 mM NaCl without glucose or to 250 mM NaCl plus glucose with 100 μM DCCD (pH 7.1) failed to swell at 20°C during a 4-h period. Presumably the cells are relatively impermeable to sodium at the lower temperature.

Effect of ionophores on swelling. Several ionophores were utilized to selectively dissipate either the chemical or electrical components of the proton motive force. Nigericin, by promoting electroneutral K^+-H^+ exchange (8, 23), collapsed the pH gradient in the presence of 4.3 mM external KCl (Table 1) or 100 mM KCl (data not shown). The K^+ ionophore valinomycin (23) collapsed the electrical potential in the presence of high external potassium. The combination of valinomycin and nigericin induced rapid swelling of cells incubated in NaCl and glucose with 100 mM KCl (Fig. 6). Swelling was prevented by sucrose (Fig. 6). Neither nigericin nor valinomycin alone induced swelling in the presence of glucose and NaCl (data not shown). NaSCN (25 mM), which collapsed the membrane potential, acted in analogous fashion to valinomycin in the above swelling experiments. The proton ionophores carbonyl cyanide-*m*-chlorophenyl hydrazone (1 to 100 μM) and carbonyl cyanide-*p*-trifluoro-methoxyphenyl hydrazone (0.2 to 2 μM) collapsed only the chemical component of the proton motive force and failed to cause swelling in the presence of glucose and NaCl. Gramicidin (10 μM), a nonspecific cation channel (8, 23), induced rapid cell swelling and lysis in the presence of NaCl and glucose (data not shown). None of the above ionophores affected the production of ATP by glycolysis. It would thus appear that the presence of either a pH gradient or a membrane potential can provide the energy to maintain a cell volume.

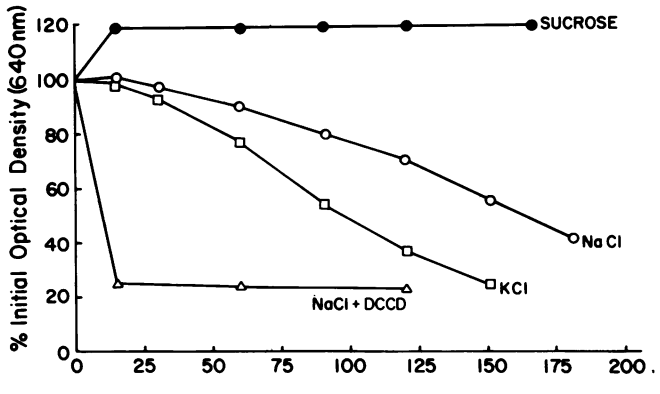

FIG. 5. Swelling in NaCl, KCl, and sucrose. Cells were grown to an optical density at 640 nm of 0.27 (pH 6.6), washed once in 250 mM NaCl–10 mM *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (pH 7.4), and suspended in 20 ml of same solution. A sample (0.15 ml) of these cells was added to a final volume of 5 ml (0.15 mg of cell protein per ml) and incubated at 37°C in the following: 0.5 M sucrose (●), 250 mM NaCl (○), 250 mM KCl (□), or 250 mM NaCl plus 200 μM DCCD (△). The optical density was followed at 640 nm.

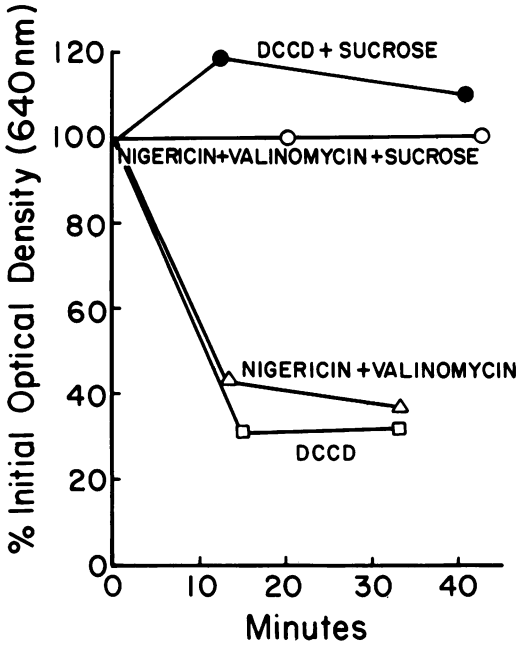

FIG. 6. Effect of ionophores on swelling. Cells were grown to an optical density at 640 nm of 0.32 (pH 6.6), washed once in 250 mM NaCl–50 mM *N*-2-acetamido-2-aminoethanesulfonic acid (pH 6.5), and suspended in 5 ml of the same buffer. A sample (0.2 ml) of cells was added to give final volume of 2 ml containing 10 mM glucose, 100 mM KCl, 50 mM *N*-2-acetamido-2-aminoethanesulfonic acid (pH 6.5), and the following: 250 μM DCCD plus 0.5 M sucrose (●); 250 μM DCCD plus 250 mM NaCl (□); 15 μM nigericin plus 7.5 μM valinomycin and 0.5 M sucrose (○); or 15 μM nigericin plus 7.5 μM valinomycin and 250 mM NaCl (△).

Correlation of the pH gradient and volume in the presence of DCCD. The results presented in Fig. 3 demonstrated a correlation between cell swelling and a decrease in ATP (and thus proton motive force). To further examine the relationship between cell volume and proton motive force, a study was made of the time course of swelling and proton motive force in the presence of DCCD. An external pH of 5.5 was chosen because under these conditions the pH gradient is the major component of the proton motive force (27). Incubation of cells in NaCl plus DCCD led to a rapid fall in the pH gradient over the first 2 min and then a slower fall during the subsequent 5 min (Fig. 7). With DCCD the cells swelled continuously during the experimental period. Lysis did not

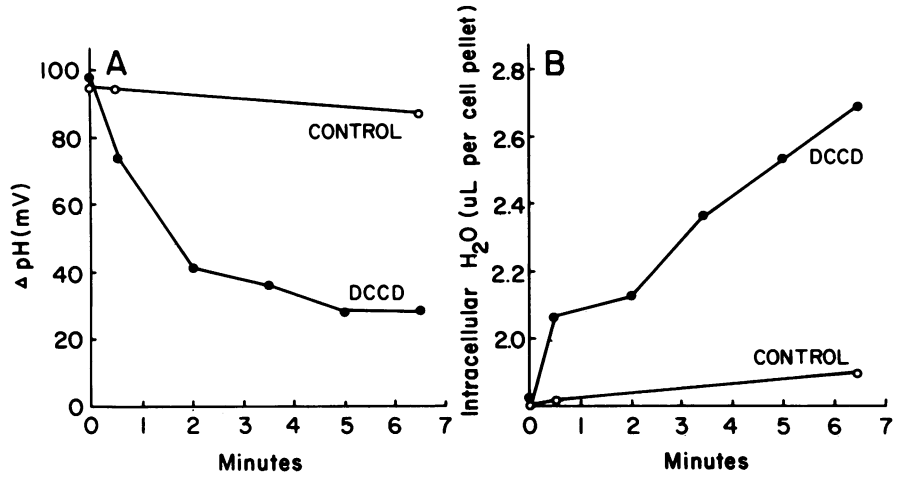

FIG. 7. Effect of DCCD on ΔpH and cell swelling. Cells were grown to an optical density at 640 nm of 0.17 (pH 7.0) and washed once in 250 mM NaCl–50 mM 2-(N-morpholino)ethanesulfonic acid (pH 5.5). Cells were suspended in the same buffer at approximately 1.0 mg of cell protein per ml. (A) To a portion of stock cells was added 10 mM glucose, [^{14}C]benzoic acid (0.04 μCi/ml, 0.4 μg/ml), and either 250 μM DCCD (●) or ethanol (0.25% final concentration) (○). The ΔpH was determined as described in the text. (B) Intracellular water was determined for cells incubated under identical conditions (but without benzoic acid) with DCCD (●) or ethanol (○) as described in the text.

occur during the experiment, since the cells remained impermeable to inulin. DCCD-treated and control cells had nearly identical levels of ATP throughout the experiment. Thus, there was a close relationship between the fall of the pH gradient (proton motive force) and the increase in cellular volume.

Correlation between ATP and cell volume. An additional experiment was performed to explore further the relationship between ATP concentration and cell volume. The non-metabolizeable glucose analog, α-methylglucoside, a substrate of the glucose group translocation system (4), was used to alter the intracellular ATP concentration (1). Over the range of ATP concentrations between 0.1 and 1.7 mM the cell volume was maintained at a constant level. This is consistent with the observation (Fig. 3) that the ATP concentration must fall to very low levels before swelling begins.

DISCUSSION

Cells of *M. gallisepticum* swell and lyse when suspended in 250 mM NaCl solutions in the absence of an energy source. This is due to the inward diffusion of NaCl (and water) as a result of the Gibbs-Donnan forces generated by intracellular anionic macromolecules. This view is supported by the direct demonstration of Na^+ entry (15) and by the observation that nondiffusable molecules such as disaccharides and $MgSO_4$ completely prevent swelling. In normal cells provided with a source of energy, the unavoidable inward diffusion of NaCl must be exactly balanced by an outward extrusion by some type of mechanism. The energy dependence of the NaCl extrusion is clearly shown by the effect of glucose in preventing the swelling process. Furthermore, the addition of glucose after swelling has begun results in shrinkage of the cell back to the original volume. The rise in intracellular ATP concentration with the addition of glucose is correlated with an elevation of the proton motive force consisting of a pH gradient (inside alkaline) and a membrane potential (inside negative).

The essential role of an ATP-driven cation pump is indicated by the profound effect of the ATPase inhibitor DCCD. The addition of DCCD to energized cells results in rapid swelling in spite of high levels of ATP. This swelling is also prevented by sucrose or $MgSO_4$, indicating that in the DCCD experiment Gibbs-Donnan forces are unopposed by NaCl extrusion mechanisms.

There are two alternative roles for the ATPase that may be considered. The first possibility involves a proton-translocating ATPase, which generates a proton motive force, which in turn energizes an Na^+-H^+ exchange mechanism. A second mechanism involves an ATP-driven Na^+ pump. The available evidence favors the former hypothesis. A proton motive force of 60 to 130 mV has been observed across the plasma membrane (27). DCCD blocks both the ΔpH and the ΔΨ, implying that the ATPase is responsible for both ΔpH and ΔΨ. In the accompanying communication (15), direct measurement of Na^+ and H^+ movements provide support for the existence of an Na^+-H^+ antiport reaction. If such an antiport were electrogenic (Na^+-$2H^+$, for example) either ΔΨ or ΔpH could drive Na^+ exit, consistent with the ionophore experiments of Fig. 6.

The finding that the proton ionophores collapse the ΔpH, but not the ΔΨ, and fail to cause swelling is not entirely understood. Although carbonyl cyanide-*m*-chlorophenyl hydrazone collapses both ΔΨ and ΔpH in most organisms (30), effects on ΔpH alone have been reported in *Mycoplasma mycoides* var. Capri (13, 14). Benyoucef et al. (2) reported that carbonyl cyanide-*p*-trifluoro-methoxyphenyl hydrazone was able to collapse the membrane potential of *M. mycoides* at pH 5.6, but not at pH 7.3. Apparently other ions are involved in the maintenance of the ΔΨ in the presence of the ionophore.

Although our current knowledge of the mechanism of volume regulation remains incomplete, all of the evidence available is consistent with the view that *M. gallisepticum* extrudes NaCl (and water) by a combination of a proton-translocating ATPase and an electrogenic Na^+-H^+ exchange carrier (with more than one H^+ per Na^+).

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AM-05736 from the National Institutes of Health and by National Science Foundation grant PCM-81-17278.

LITERATURE CITED

1. **Benyoucef, M., J.-L. Rigaud, and G. Leblanc.** 1981. Gradation of the magnitude of the electrochemical proton gradient in Mycoplasma cells. Eur. J. Biochem. **113**:499–506.
2. **Benyoucef, M., J.-L. Rigaud, and G. Leblanc.** 1982. Cation transport mechanisms in *Mycoplasma mycoides* var. Capri cells: Na^+-dependent K^+ accumulation. Biochem. J. **208**:529–538.
3. **Benyoucef, M., J.-L. Rigaud, and G. Leblanc.** 1982. Cation transport mechanisms in *Mycoplasma mycoides* var. Capri cells: the nature of the link between K^+ and Na^+ transport. Biochem. J. **208**:539–547.
4. **Cirillo, V. P.** 1979. Transport sytems, p. 323–349. *In* M. F. Barile and S. Razin (ed.), The mycoplasmas, vol. 1. Academic Press, Inc., New York.
5. **Cole, H. A., J. W. T. Wimpenny, and D. E. Hughes.** 1967. The ATP pool in *Escherichia coli*. I. Measurement of the pool using a modified luciferase assay. Biochim. Biophys. Acta **143**: 445–453.
6. **DeKruyff, B., A. Demel, and L. L. M. VanDeenan.** 1972. The effect of cholesterol and epicholesterol incorporation on the permeability and on the phase transition of intact *Acholeplasma laidlawii* cell membranes and derived liposomes. Biochim. Biophys. Acta **255**:331–347.
7. **Epstein, W., and L. Laimins.** 1980. Potassium transport in *Escherichia coli*: diverse systems with common control by osmotic forces. Trends Biochem. Sci. **5**:21–23.
8. **Harold, F. M., and K. Altendorf.** 1974. Cation transport in bacteria: K^+, Na^+ and H^+, p. 1–50. *In* F. Bronner and A. Kleinzeller (ed.), Current topics in membranes and transport. Academic Press, Inc., New York.
9. **Jacobs, M. H., and D. R. Stewart** 1947. Osmotic properties of the erythrocyte. XII. Ionic and osmotic equilibria with a complex external solution. J. Cell. Comp. Phys. **30**:79–103.
10. **Jinks, D. C., J. R. Silvius, and R. N. McElhaney.** 1978. Physiological role and membrane lipid modulation of the membrane-bound (Mg^{2+}, Na^+) adenosine triphosphatase activity in *Acholeplasma laidlawii*. J. Bacteriol. **136**:1027–1036.
11. **Leaf, A.** 1956. On the mechanism of fluid exchange of tissues *in vitro*. Biochem. J. **62**:241–248.
12. **Leaf, A.** 1959. Maintenance of concentration gradients and regulation of cell volume. Ann. N.Y. Acad. Sci. **72**:396–404.
13. **Leblanc, G., and C. LeGrimellec.** 1979. Active K^+ transport in *Mycoplasma mycoides* var. Capri: relationships between K^+ distribution, electrical potential, and ATPase activity. Biochim. Biophys. Acta **554**:168–179.
14. **LeGrimellec, C., D. Lajeunesse, and J.-L. Rigaud.** 1982. Effects of energization on membrane organization in mycoplasma. Biochim. Biophys. Acta **687**:281–290.
15. **Linker, C., and T. H. Wilson.** 1985. Sodium and proton transport in *Mycoplasma gallisepticum*. J. Bacteriol. **163**:1250–1257.
16. **Linker, C., and T. H. Wilson.** 1985. Characterization and solubilization of the membrane-bound ATPase of *Mycoplasma gallisepticum*. J. Bacteriol. **163**:1258–1262.
17. **Macknight, A. D. C., and A. Leaf.** 1977. Regulation of cellular volume. Physiol. Rev. **57**:510–573.
18. **Macknight, A. D. C., and A. Leaf.** 1978. Regulation of cellular volume, p. 315–334. *In* T. E. Andreoli, J. F. Hoffman, and D. D. Fanestil (ed.), Physiology of membrane disorders. Plenum Publishing Corp., New York.
19. **Maloney, P. C., E. R. Kashket, and T. H. Wilson.** 1975. Methods for studying transport in bacteria. Methods Membr. Biol. **5**:1–49.
20. **Patterson, M. S., and R. C. Greene.** 1965. Measurement of low energy beta-emitters in aqueous solution by liquid scintillation counting of emulsions. Anal. Chem. **37**:854–857.
21. **Peterson, G. L.** 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. **83**:346–356.
22. **Pollack, J. D., V. V. Tryon, and K. D. Beaman.** 1983. The metabolic pathways of acholeplasma and mycoplasma: an overview. Yale J. Biol. Med. **56**:709–716.
23. **Pressman, B. C.** 1976. Biological applications of ionophores. Annu. Rev. Biochem. **45**:501–530.
24. **Razin, S.** 1964. Factors influencing osmotic fragility of Mycoplasma. J. Gen. Microbiol. **36**:451–459.
25. **Razin, S.** 1979. Membrane proteins, p. 289–322. *In* M. F. Barile and S. Razin (ed.), The mycoplasmas, vol. 1. Academic Press Inc., New York.
26. **Razin, S., and S. Rottem.** 1976. Techniques for the manipulation of mycoplasma membranes, p. 3–26. *In* A. H. Maddy (ed.), Biochemical analysis of membranes. Chapman and Hall, Inc., London.
27. **Rottem, S., C. Linker, and T. H. Wilson.** 1981. Protonmotive force across the membrane of *Mycoplasma gallisepticum* and its possible role in cell volume regulation. J. Bacteriol. **145**:1299–1304.
28. **Rottem, S., and A. J. Verkleij.** 1982. Possible association of segregated lipid domains of *Mycoplasma gallisepticum* membranes with cell resistance to osmotic lysis. J. Bacteriol. **149**:338–345.
29. **Shirvan, M. H., Z. Gross, Z. Ne'eman, and S. Rottem.** 1982. Isolation of *Mycoplasma gallisepticum* membranes by a mild alkaline-induced lysis of nonenergized cells. Curr. Microbiol. **7**:367–370.
30. **Simoni, R. D., and P. W. Postma.** 1975. The energetics of bacterial active transport. Annu. Rev. Biochem. **44**:523–554.
31. **Smith, P. F., and S. Sasaki.** 1958. Stability of pleuropneumonia-like organisms to some physical factors. Appl. Microbiol. **6**:184–189.
32. **Tosteson, D. C.** 1964. Regulation of cell volume by sodium and potassium transport, p. 3–22. *In* J. F. Hoffman (ed.), The cellular functions of membrane transport. Prentice-Hall, Inc., Englewood Cliffs, N.J.
33. **Tosteson, D. C., and J. F. Hoffman.** 1960. Regulation of cell volume by active cation transport in high and low potassium sheep red cells. J. Gen. Physiol. **44**:169–194.
34. **Wilbrandt, W.** 1941. Osmotische natur sogenannter nichtosmotischer Hamolysen (Kolloidosmotische Hamolyse). I. Mitteilung. Arch. Ges. Physiol. **245**:22–52.
35. **Wilson, T. H.** 1954. Ionic permeability and osmotic swelling of cells. Science **120**:104–105.