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Magnesium and Manganese Content of Halophilic Bacteria

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Magnesium and manganese contents were measured by atomic absorption spectrophotometry in bacteria of several halophilic levels, in *Vibrio costicola*, a moderately halophilic eubacterium growing in 1 M NaCl, *Halobacterium volcanii*, a halophilic archaebacterium growing in 2.5 M NaCl, *Halobacterium cutirubrum*, an extremely halophilic archaebacterium growing in 4 M NaCl, and *Escherichia coli*, a nonhalophilic eubacterium growing in 0.17 M NaCl. Magnesium and manganese contents varied with the growth phase, being maximal at the early log phase. Magnesium and manganese molalities in cell water were shown to increase with the halophilic character of the logarithmically growing bacteria, from 30 mmol of Mg per kg of cell water and 0.37 mmol of Mn per kg of cell water for *E. coli* to 102 mmol of Mg per kg of cell water and 1.6 mmol of Mn per kg of cell water for *H. cutirubrum*. The intracellular concentrations of manganese were determined independently by a radioactive tracer technique in *V. costicola* and *H. volcanii*. The values obtained by ^{54}Mn loading represented about 70% of the values obtained by atomic absorption. The increase of magnesium and manganese contents associated with the halophilic character of the bacteria suggests that manganese and magnesium play a role in haloadaptation.

Halobacteria represent a unique class of microorganisms that are adapted to extreme environmental conditions. The use of the 16S rRNA sequences to study molecular evolution has introduced a revolution in bacterial taxonomy, whereby the halobacteria are placed along with methanogens and thermoacidophiles in a new kingdom, the *Archaebacteria*, which separated after the beginning of life from the other procaryotes called *Eubacteria* and the eucaryotes (25, 26). Halobacteria are adapted to a niche containing waters of high salt concentrations under conditions of high light intensity and low oxygen tension. In the genus *Halobacterium* two kinds of organisms have been found, one including *Halobacterium cutirubrum* and the other including *Halobacterium volcanii*. The bacteria related to *H. cutirubrum* grow in 4 M sodium chloride, contain gas vacuoles and synthesize a purple membrane which can use light as a source of energy (16). The bacteria related to *H. volcanii* are devoid of gas vacuoles and purple membrane and grow in a medium containing lower sodium chloride concentrations (2.5 M) but higher magnesium concentrations (0.25 to 1.5 M) (3). Moderately halophilic bacteria are found outside the *Archaebacteria*. Such is the case of *Vibrio costicola*, which was isolated from bacon brines and requires 1 M sodium chloride for optimum growth (6).

Halophilic bacteria must possess novel mechanisms of ionic balance to cope with their extreme ionic environments. The *Archaebacteria* are of particular interest in this regard because they may have diverged from *Eubacteria* when mechanisms of ionic balance were evolving de novo. Our current understanding of ionic regulation in halophilic bacteria is fragmentary. In previous studies devoted to determining the intracellular concentrations of the monovalent cations K^+ and Na^+ in halophilic bacteria, it was found that these cells regulated their internal osmotic pressure by controlling their intracellular potassium concentrations (2, 7, 19). Intracellular concentrations of divalent cations in halophilic bacteria have not been so well studied. In one study, *V. costicola* had an intracellular magnesium concentration of 48 mM, which was of the same order of magnitude as in other *Eubacteria* (20). To further characterize ionic regulation in halophilic bacteria we examined their Mg^{2+} and Mn^{2+} contents, which are structurally and metabolically important ligands of nucleic acids, proteins, peptidoglycans, and lipids.

MATERIALS AND METHODS

Bacterial growth. *H. cutirubrum* 34001 from the National Research Council of Canada (Ottawa) was grown on a complex medium (11) modified to include glycerol, which increased the growth yield (12). The composition of the culture medium was 7.5 g of Casamino Acids (Difco Laboratories), 10 g of yeast extract, 3 g of sodium citrate, 2 g of KCl, 20 g of $MgSO_4 \cdot 7H_2O$, 250 g of NaCl, 0.23 g of $FeCl_3$, and 8.3 ml of glycerol per liter of water. The pH was adjusted at 6.5 with NaOH, and the medium was autoclaved for 20 min at 125°C and 15 lb/in^2 pressure. The culture was illuminated with floodlights and incubated in a rotatory water bath at 37°C.

H. volcanii 2112 from the National Research Council of Canada (Ottawa) was grown, as recommended, at 37°C, on a sterile medium containing 5 g of tryptone, 3 g of yeast extract, 1.34 g of $CaCl_2 \cdot 2H_2O$, 10 g of KCl, 10 g of $MgSO_4 \cdot 7H_2O$, 45 g of $MgCl_2 \cdot 6H_2O$, and 125 g of NaCl per liter of water.

V. costicola 37001 from the National Research Council of Canada (Ottawa) was grown on a complex medium at 1 M NaCl (6). The culture medium contained 5 g of Proteose Peptone, 5 g of tryptone, and 58 g of NaCl per liter of water. The medium was adjusted at pH 7.5 with NaOH and autoclaved. Cells were grown at 37°C with vigorous aeration.

Escherichia coli K-12 was grown on L broth at 37°C. All of the cultures were monitored for their A_{660} with a Cary 118C spectrophotometer.

Quantitation of bacteria. For each bacterial strain a correlation was established between the turbidity of the culture measured by its A_{660}, the number of cells counted under the microscope, the dry weight of the cells, and the protein content of the cells.

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Protein measurements were made by the method of Bradford (1) with a protein assay kit (Bio-Rad Laboratories). The protein coloration was measured at 595 nm on a Cary 118C spectrophotometer. The results were expressed as milligrams of protein per gram of cell dry weight, using bovine serum albumin as a standard.

The bacteria were counted on microphotographs of a Petroff-Hauser chamber, which were taken with the photomicroscope II from Carl Zeiss, and the statistical error was evaluated by the method of Miale (14). Before the counting of *V. costicola*, the bacteria were detached with sucrose (8). The bacteria were washed in 1 M NaCl, suspended in 0.5 M sucrose at pH 7.8, and incubated under gentle agitation at 25°C for 1 h. At that time, the bacteria appeared detached under the microscope and could be counted. The spheroplasts were checked for integrity by dialysis of the sucrose solution against 1 M NaCl. During the sucrose treatment, the bacterial suspension was characterized by a 10 to 30% increase of the A_{660}. During the dialysis process, the bacterial suspension returned to its original absorbance, which indicated that the cells, swollen in sucrose, had returned to their original volume in isotonic salt solution. About 17% of the spheroplasts were viable when plated on agar.

Evaluation of cell water. Cell water was evaluated by the method of Christian and Waltho (2). Bacteria were centrifuged from culture in preweighed, stoppered polycarbonate bottles at 4,500 × *g* for 20 min (Beckman 50 Ti rotor). The bacteria were washed twice by centrifugation in the same conditions of suspensions of 70 to 100 mg of wet cells in 6 ml of isotonic solution containing NaCl at 10 g/liter for *E. coli*, 58 g/liter for *V. costicola*, 167 g/liter for *H. volcanii*, and 234 g/liter for *H. cutirubrum*. Owing to the fragility of halophilic bacteria, bacterial material was released in the supernatant at higher centrifugal forces. The optimum centrifugal force of 4,500 × *g* was determined as the highest *g* value for which the supernatant was devoid of measurable absorbance at 260 nm. In a review about cell water determination (18), Rothstein reported values of interstitial fluid space which represented less than 26% of the total fluid volume of the pellet, i.e., less than the interstitial volume of packed spheres. We could not obtain similar figures because of the centrifugal limitations, and we observed interstitial fluid spaces which represented 45 to 66% of the total fluid volume of the pellet (see Table 2). After the final centrifugation, the supernatants were drained, the walls of the bottles were carefully rinsed and patted dry with cotton tips, and the bottles were weighed before and after desiccation in an oven at 100°C during 24 h.

The wet and dry weights of the pellets so determined result from the wet and dry weights of the cells plus the wet and dry weights of the interstitial fluid. The interstitial fluid space was measured by the method of Takacs et al. (24). Each wet pellet was suspended in 1 ml of isotonic solution containing about 10^6 cpm of [^{14}C]inulin (New England Nuclear Corp.; 2.4 mCi/g), with gentle mechanical agitation. The bottles were centrifuged at 4,500 × *g*, 100 μl of each supernatant was diluted in 5 ml of Scintiverse II (Fisher Scientific Co.), and the radioactivity was measured by scintillation counting. The rest of the supernatant was drained with the same precautions taken previously. The pellets were resuspended in 1 ml of cold isotonic solution and centrifuged again. The radioactivity of 100 μl of the second supernatant was also determined. The dilution of the radioactive nonpenetrating solute inulin allowed the calculation of the interstitial fluid volume trapped in the pellet. After [^{14}C]inulin treatment, wet and dry weights of the pellets were measured, and no significant loss of bacterial material was observed.

The cell water values were calculated from the relations: pellet wet weight = cell wet weight + $X(C_s + C_w)$ and pellet dry weight = cell dry weight + XC_s, where the weights were expressed in grams, X was the interstitial fluid space expressed in milliliters, and C_s and C_w were, respectively, the anhydrous solute concentration and the water concentration in grams per milliliter of the isotonic solutions (27). The total water of the pellet is the wet weight of the pellet minus the dry weight of the pellet. Since the total water equals the sum of the cell water and of the interstitial water, then the cell water (milliliters per gram of cell dry weight) is given by: (pellet wet weight − pellet dry weight − XC_w)/(pellet dry weight − XC_s).

The correction introduced for the water concentration was not negligible in the case of halophilic bacteria. C_w values were, respectively, 0.995, 0.980, 0.942, and 0.916 g/ml in the isotonic solutions of *E. coli*, *V. costicola*, *H. volcanii*, and *H. cutirubrum*.

Mn and Mg analysis. The glass tubes used for the mineralization of the samples were washed with hot ultrapure 70% HNO_3 and thoroughly rinsed with deionized water to avoid any metal contamination. The bacteria were collected by centrifugation at different growth phases, washed in isotonic saline, and oven dried. Because the medium of *H. cutirubrum* contains magnesium, we monitored the washing procedure until no significant loss of metal occurred. Samples of dry bacteria (about 250 mg) were oxidized with 1 ml of ultrapure 65 to 70% HNO_3 and 0.5 ml of ultrapure 70% $HClO_4$ for 24 h at 40°C. Ultrapure concentrated H_2SO_4 (25 μl) was added to avoid the formation of volatile metallic complexes with perchloric acid. The tubes were then dried on a hot plate for 6 to 8 h. The ashes were redissolved in 10 ml of 1% nitric acid. Dilutions and standards were prepared with 1% nitric acid.

Manganese was analyzed by atomic absorption spectrophotometry on a Perkin-Elmer 370A apparatus at 279.5 nm, equipped with an HGA 2100 electrothermal atomization furnace and an AS-1 autosampler. Pyrolysis-coated tubes and argon gas were used throughout.

Magnesium was analyzed by atomic absorption spectrophotometry on a Perkin-Elmer 360 apparatus at 283.6 nm with an air-acetylene flame.

Accumulation of intracellular radioactive manganese. Radioactive ^{54}Mn was used to measure the intracellular manganese accumulation in *V. costicola* and *H. volcanii* by the method of Eisenstadt et al. (5). The two strains displayed a saturable accumulation of the manganese tracer. The internal manganese content of logarithmically growing cells followed the external manganese concentrations as a Langmuir adsorption isotherm: $[Mn]_{in} = [Mn]_{max} \times [Mn]_{out}/(K + [Mn]_{out})$, where $[Mn]_{in}$ is the internal manganese content at equilibrium, $[Mn]_{out}$ is the extracellular concentration, $[Mn]_{max}$ is the capacity or maximum amount the cells could accumulate, and K is the Michaelis-Menten half-saturation constant for the equilibrium levels of accumulation of manganese by the transport system.

^{54}Mn (0.4 μCi/ml, carrier free; New England Nuclear) raised the extracellular manganese concentration a negligible 10^{-12} M, and its addition was ignored in the calculations. Manganese was undetectable in the culture media by atomic absorption spectrophotometry, and therefore its presence in the media in trace quantities was also ignored in the calculations.

TABLE 1. Manganese and magnesium contents

Species	Generation time at 37°C	Phase	Mg (mg/g [dry wt] of pellet)	Mn (μg/g [dry wt] of pellet)	Mg/Mn molar ratio
E. coli	22 ± 0.6 min	Early log	2.33 ± 0.06	37.2 ± 0.9	142
		Late log	1.32 ± 0.05	13.3 ± 0.05	224
		Stationary	0.86 ± 0.01	7.31 ± 0.35	266
V. costicola	86.0 ± 3.6 min	Early log	1.18 ± 0.04	40.3 ± 0.8	66
		Late log	0.36 ± 0.01	7.00 ± 0.01	116
		Stationary	0.53 ± 0.01	12.3 ± 0.06	97
H. volcanii	9.0 ± 0.2 h	Early log		50.6 ± 1.7	
		Late log			
		Stationary			
H. cutirubrum	27.4 ± 0.8 h	Early log	1.22 ± 0.03	43.1 ± 0.2	64
		Late log	1.45 ± 0.02	45.0 ± 0.9	73
		Stationary	1.34 ± 0.02	27.3 ± 0.08	111

About 10^8 cells were incubated at 25°C with defined concentrations of cold $MnCl_2$ and 0.4 μCi of ^{54}Mn per ml. At fixed periods of time, 20-μl samples were filtered (HA filters; Millipore Corp.) and washed two times with 5 ml of isotonic salt solution to remove unfixed radioactivity. The filters were then dissolved in vials containing 10 ml of Scintiverse II, and the radioactive manganese associated with the cells was determined in a Beckman model LS-8000 scintillation counter (22).

Blanks were run without bacteria, in the same experimental conditions, showing that at a pH below 5.5 the radioactive manganese was not retained on the filter, but that at pH 5.8 and above a fraction of the total radioactivity was retained on the filter, presumably as a result of nonenzymatic oxidation by components of the growth medium (17). We therefore conducted the accumulation assays at pH 5.5, the maximal pH at which manganese cations appeared completely soluble.

Controls were also made with bacteria incubated with radioactive manganese and then lysed by hypotonic shock in pure water. Only background radioactivity remained associated with the cell debris after extensive washing, indicating that the radioactive manganese retained on the filter with intact bacteria was associated with the cells and did not represent manganese removed from solution by biooxidation (4).

RESULTS

The manganese and the magnesium contents were measured in *E. coli*, *V. costicola*, *H. volcanii*, and *H. cutirubrum* at different growth phases (Table 1). The growth curve of each organism was followed at 660 nm in four cultures, and the generation times were calculated from the semilogarithmic plots of absorbance against time. Ten samples were taken at each growth phase, the cells were reduced to ashes, and the manganese and magnesium contents were measured. In *H. cutirubrum*, the magnesium content was fairly constant, whereas in *E. coli* and *V. costicola* the manganese and the magnesium contents declined as growth progressed. It was meaningless to compare strains with regard to metallic contents per gram of dry weight of pelleted bacteria since different strains could contain different amounts of solid material in the interstitial space, especially since the halophilic bacteria were bathed in high concentrations of salts. However, magnesium/manganese molar ratios in each strain could be taken to indicate their relative abundances, and it appeared that the halophilic bacteria *V. costicola* and *H. cutirubrum* contained proportionally more manganese than did *E. coli* in all growth phases.

Intracellular manganese and magnesium values were expressed as apparent molalities, i.e., as millimoles per kilogram of cell water, on the assumption that the solutes were in free solution and were equally distributed throughout the cell water. These rough estimations of intracellular magnesium and manganese contents must be taken with caution and were only presented for the purpose of comparison with published values obtained by this procedure. In fact the methods used for the measurement of the cations did not distinguish between bound and free cations. Measurements of wet weights, dry weights, and interstitial volumes in bacterial pellets allowed the calculation of the cell water (see Materials and Methods), which was expressed per gram of cell dry weight (Table 2). The measurements were carried out for each strain in the logarithmic growth phase with at least 10 samples of bacteria from three separate cultures. The cells were counted in six batches for each bacterial strain at a minimum of five different dilutions, and the counts per A_{660} unit were derived from a regression line of the

TABLE 2. Composition of cell material

Species	Wet wt/dry wt ratio of pelleted bacteria	Interstitial space (ml/g of pellet wet wt)	Interstitial water (% of total)	Cell water (g/g of cell dry wt)	Protein (mg/g of cell water)	Cell water (pg per cell)
E. coli	4.35	0.35 ± 0.03	45	1.85 ± 0.04	341 ± 12	4.1
V. costicola	3.82	0.43 ± 0.05	57	1.35 ± 0.02	255 ± 9	5.2
H. volcanii	2.66	0.37 ± 0.03	56	0.88 ± 0.05	248 ± 12	1.0
H. cutirubrum	2.45	0.43 ± 0.05	66	0.65 ± 0.12	127 ± 15	6.3

experimental data. Protein content was lower in *H. cutirubrum* than in the other strains, an observation which we attribute to the fact that proteins of extreme halophilic bacteria contain fewer aromatic amino acids, which are responsible for the color development of the assay, than other bacteria (13). Cell counting and the evaluation of the cell water allowed the calculation of the cell water per cell. The values of cell water per individual cell, which were in the order of 1 μm^3, correlated well with the form of the bacteria under the microscope: *H. volcanii* looked shrunken under the microscope, and *E. coli*, *V. costicola*, and *H. cutirubrum* constituted a series of more and more elongated forms (Fig. 1).

From the data on cell water and metal content, apparent magnesium and manganese molalities were calculated at the logarithmic growth phase for *E. coli*, *V. costicola*, *H. volcanii*, and *H. cutirubrum* (Table 3). When the results were expressed in this way, a clear correlation could be seen between the intracellular concentrations of Mg and Mn and the ionic strength of the natural environment of the bacteria. We plotted the intracellular molalities of magnesium and manganese of the bacteria as a function of the sodium chloride molalities of the isotonic water solutions (Fig. 2). The lower molar ratio of magnesium to manganese in halophilic organisms (Table 1) was supported by the fact that the increase of intracellular manganese with the extracellular ionic strength was proportionally greater than the increase of intracellular magnesium (Fig. 2).

We used an independent method to assess the intracellular concentrations of manganese, based on the accumulation of radioactive manganese, in *V. costicola* and *H. volcanii* (see Materials and Methods). We studied the time course of the accumulation of ^{54}Mn to select the time interval necessary to measure the equilibrium values of the radioactive pool (Fig. 3). The growth of the culture was negligible compared with the equilibration time. The generation time of *V. costicola* and *H. volcanii* was determined at 37°C and at neutral pH (Table 1). The bacteria were still viable but their growth rates were much slower in the conditions of the accumulation assay, at 25°C and at acidic pH. The majority of the accumulated radioactive manganese was in an easily mobilized state, since the chase of the accumulated pool with cold manganese was rapid and left, respectively, 10 and 17% of residual radioactivity in *V. costicola* and *H. volcanii* (Fig. 3). We investigated further the mechanism of manganese accumulation, which was inhibited at low temperature and by sodium cyanide and dinitrophenol. Cells of *V. costicola* and *H. volcanii* treated with 5 mM dinitrophenol did not accumulate radioactive manganese. When the cells were first loaded with the radioactive tracer, the subsequent addition of 5 mM dinitrophenol did not cause a decrease of the amount of the tracer associated with the cells, indicating that the effect of dinitrophenol was not due to the formation of a manganese complex unable to be adsorbed on or taken up by the cells (Fig. 3). Moreover, in cells loaded with radioactive manganese which were treated successively at a 1-min interval with 5 mM dinitrophenol and 1 mM cold manganese, there was no chase of the radioactive tracer by cold manganese, indicating that dinitrophenol inhibits the exchange of manganese between two separate compartments, the extracellular solution and the cells (Fig. 3).

We measured the equilibrium concentrations of ^{54}Mn at different extracellular manganese concentrations, and from the hyperbolic dependence we calculated the Langmuir constant and the manganese capacity (Fig. 4). The manganese capacities obtained by this method for *V. costicola* (0.447 mmol/kg of cell water) and *H. volcanii* (0.864 mmol/kg of cell water) represented, respectively, 74 and 68% of the values determined by atomic absorption spectrophotometry. The lack of absolute agreement between the two methods could be attributed to the presence of a pool of manganese that was inaccessible to the radioactive tracer and thus could not be measured by the tracer method, but was only detected when total manganese was measured by atomic absorption.

DISCUSSION

We measured, by atomic absorption, magnesium and manganese associated with the halophilic bacteria *V. costicola*, *H. volcanii*, and *H. cutirubrum* and with the nonhalophilic bacterium *E. coli* for comparison. The values reported in Table 1 represent the total metal contents associated with the cells. Two important methodological considerations in this work are the procedures for washing the cells and for extracting magnesium and manganese from them. It was important to process the bacteria by a method preventing extracellular metal contamination, since the archaebacteria *H. cutirubrum* and *H. volcanii* were grown, respectively, in the presence of 0.08 and 0.26 M magnesium. To prevent nonspecific adsorptions on bacteria, some researchers wash them with demineralized water (10). On the contrary, others avoid the washing procedure and measure the metal content directly with the culture medium to preserve the intracellular-extracellular charge and osmotic equilibria, which may influence the retention of metals (9). Since in our case it was necessary to wash the medium, we adopted an extensive washing procedure with isotonic salines, suitable for halophiles, which are known to lyse in pure water (20). The extraction of magnesium and manganese from the cells was achieved by the most efficient method, the hot acid extraction method, to ensure the proper recovery of the divalent cations, even when tightly bound (23).

We found an effect of growth phase on magnesium and manganese contents of the cells. Magnesium and manganese contents decreased at the late logarithmic and stationary phases. This diminution was not observed in *H. cutirubrum*, a slow-growing archaebacterium. The ionic economy of bacteria has often been found to be related to the physiological conditions. In methanogenic bacteria, which are also slow-growing archaebacteria, an elevated content of magnesium has been observed during the stationary phase (23).

The values of intracellular molalities of magnesium and manganese are derived from the measured ionic contents of cells and the evaluation of cell water. As mentioned in Materials and Methods, the measured ratio of wet weight to dry weight of pelleted bacteria, or the measured interstitial volume in a pellet, may vary with the experimental conditions, but the cell water associated with a given dry weight of cells should be independent of the experimental conditions and should vary solely with the physiological conditions of the cells. Comparison of our values of cell water per gram of cell dry weight with those reported in the literature shows close agreement in a number of instances, but our values were somewhat lower in the case of extremely halophilic bacteria (7, 19, 20, 24). The penetration of [^{14}C]inulin, widely used for the measurement of the extracellular space of bacterial pellets, is assumed to reflect the barrier of the cytoplasmic membrane. We felt that this assumption has never been clearly tested and has been trusted only when reasonable figures of extracellular space were obtained in tightly packed bacterial pellets. Since we worked with loosely packed pellets to protect the integrity of the fragile

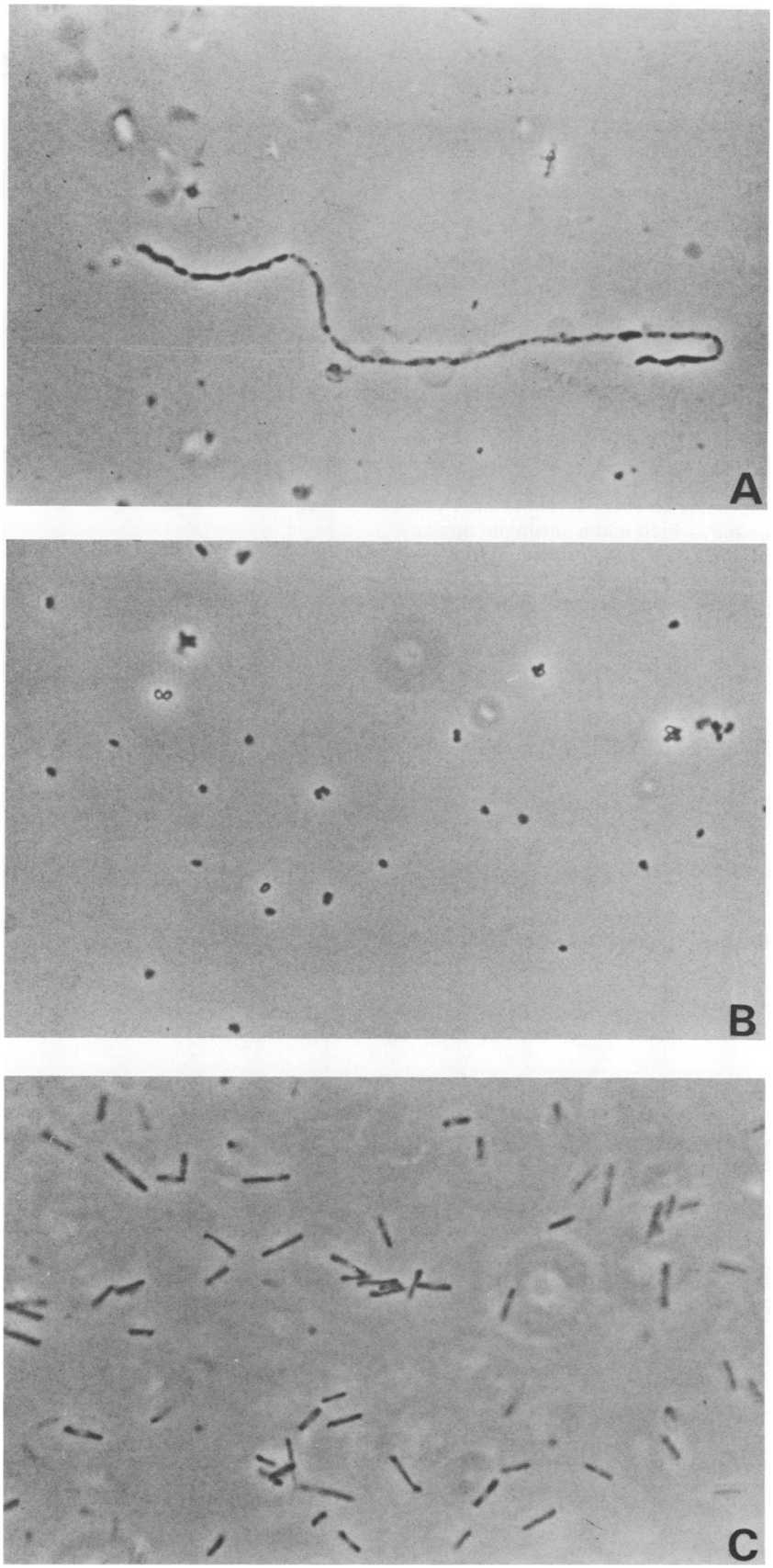

FIG. 1. Photomicrograph of halophilic bacteria at ×320 magnification: (A) *V. costicola*, (B) *H. volcanii*, (C) *H. cutirubrum*.

TABLE 3. Magnesium and manganese molalities

Species	Mg (mmol/kg of cell water)	Mn (mmol/kg of cell water)
E. coli	29.7	0.368
V. costicola	39.8	0.602
H. volcanii		1.262
H. cutirubrum	102.4	1.600

halophilic bacteria, we could not judge whether our penetration data were reasonable. We therefore developed a new method to test the penetration of [^{14}C]inulin, which consists of determining intracellular volumes in cell water per individual cell and comparing these values with the bacterial image under the microscope. The values of cell water per cell, derived from penetration of [^{14}C]inulin, can thus be tested for consistency with the apparent cell volumes under the microscope. We obtained values of cell water per individual cell ranging from 1 to 6 μm^3 which corresponded well to the morphology of the bacteria (Fig. 1; Table 2); 1 μm^3 was obtained for *H. volcanii*, which had a shrunken appearance (15); 6 μm^3 was obtained for *H. cutirubrum*, which was very elongated; and intermediate values were obtained for the coliform and vibrio, which were intermediate in size. We are confident that this new way of presenting the data is a useful method for assessing the hypothesis on the penetration of the solute used for the measurement of the extracellular space.

Magnesium is the major intracellular divalent cation in all living cells. In bacterial cells, the intracellular magnesium content has been generally found to be equivalent to 20 to 40 mM Mg. In the moderate halophile *V. costicola*, it has been reported to be 48 mM (20). Our atomic absorption values of magnesium are in agreement with the reported values (Table 3). Moreover, we have shown that in the extreme halophile *H. cutirubrum* the magnesium content is three times higher than in *E. coli*. The bacterial manganese content has been measured in several *Eubacteria* by ^{54}Mn loading (21). In logarithmically growing *Bacillius subtilis* cells, manganese has been reported to be 0.30 mmol/kg of cell water, and the

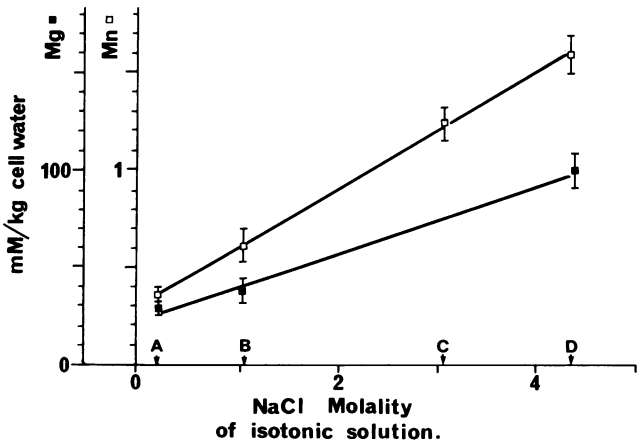

FIG. 2. Intracellular magnesium and manganese molalities in *E. coli* (A), *V. costicola* (B), *H. volcanii* (C), and *H. cutirubrum* (D) as a function of isotonic extracellular sodium molalities. Intracellular magnesium and manganese molalities have been calculated from the values of cell water per gram of cell dry weight and metal per gram of cell dry weight, which have been determined in log-phase cultures by atomic absorption spectrophotometry.

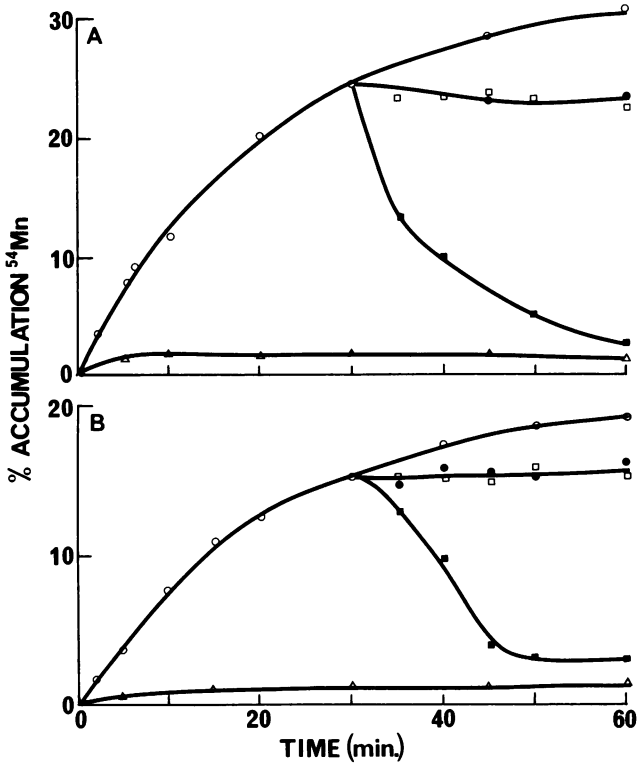

FIG. 3. Time course of accumulation of ^{54}Mn, expressed in percentage of counts per minute accumulated, in log-phase halophilic bacteria: (A) 6.2×10^7 *V. costicola* cells in the presence of 0.4 μCi of ^{54}Mn in 300 μl of culture medium at 25°C, pH 5.5; (B) 1.70×10^8 *H. volcanii* cells in the presence of 0.4 μCi of ^{54}Mn in 300 μl of culture medium at 25°C, pH 5.5. The following conditions were assayed: ○, no addition of cold manganese; △, addition of 5 mM dinitrophenol or 1 mM nonradioactive $MnCl_2$ 3 min before the start of the ^{54}Mn loading experiment; ■, addition of 1 mM nonradioactive $MnCl_2$ 30 min after the start of the ^{54}Mn loading experiment; □, addition of 5 mM dinitrophenol 30 min after the start of the ^{54}Mn loading experiment; ●, successive addition, 30 min after the start of the ^{54}Mn loading experiment, of 5 mM dinitrophenol and then, 1 min later, of 1 mM nonradioactive $MnCl_2$.

Mn/Mg molar ratio was 1:167, similar to that found in *E. coli* (21). Analysis of manganese by neutron activation in *Bacillus megaterium* yielded a value of the same order of magnitude, as far as it can be evaluated in the absence of the determination of the cell water (10). Our atomic absorption values of manganese in *E. coli* are in agreement with the reported values. Moreover, we have shown that the manganese contents of the bacteria increase with their halophilic character, and that the factor of manganese increase is greater than the corresponding factor of magnesium increase (Table 3; Fig. 2).

In the cases of *V. costicola* and *H. volcanii*, we used two independent methods for the evaluation of manganese, atomic absorption and ^{54}Mn loading. As in the other bacterial systems tested, radioactive manganese accumulation displayed the characteristics of active transport (21). The radioactive tracer was measured in cells which had been partitioned by filtration. Controls with bacteria lysed by hypotonic shock showed that no insoluble manganese was precipitated with the cells and that no radioactive manganese was associated with cell debris retained on the filter. Thus, the radioactive manganese accumulated by the cells repre-

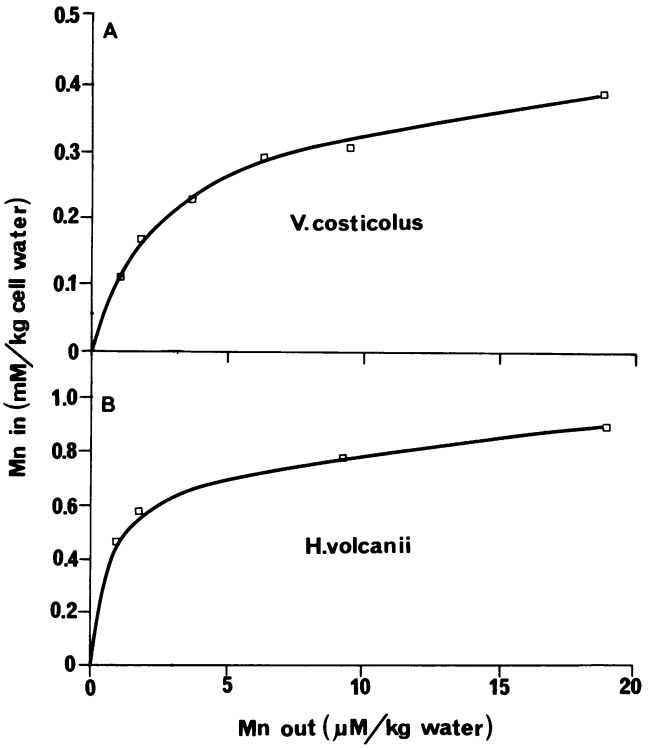

FIG. 4. Relationship between the extracellular manganese molalities and the intracellular manganese molalities in *V. costicola* (A) and *H. volcanii* (B). The equilibrium intracellular manganese molalities were evaluated by isotopic dilution of ^{54}Mn at 25°C and pH 5.5. The Langmuir isotherm relationship between $[Mn]_{out}$ and $[Mn]_{in}$ gave a K of 3.13 μmol/kg of water, and an $[Mn]_{in}$ maximum of 0.447 mmol/kg of cell water for *V. costicola* and a K of 0.91 μmol/kg of water and an $[Mn]_{in}$ maximum of 0.864 mmol/kg of cell water for *H. volcanii*.

sents truly active manganese uptake by nongrowing cells. Since our data suggest that the ^{54}Mn loading method underestimates the actual manganese content by about 30%, we suggest that the difference represents tightly bound manganese with slow exchange rates, perhaps in the cell wall.

For three different halophilic strains belonging to *Eubacteria* and *Archaebacteria* we found a general correlation between the magnesium and manganese contents and the ionic strength of the environment, suggesting that the differing divalent cationic balance in the bacteria is related to their halophilic adaptation.

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